PROTEIN-LIPID INTERACTIONS IN PLASMA MEMBRANE BIOGENESIS AND CELLULAR SIGNALING REGULATION

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Kirsten Leigh Bryant

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It is well established that protein interactions with phospholipids, particularly phosphoinositides, serve to regulate many different cellular processes. Due to the charged nature of biomembranes, electrostatic interactions are particularly effective between proteins and lipids at membrane interfaces where protein oligomerization and locally high charge densities of certain phospholipids can contribute significantly to cellular processes. Phosphoinositide distributions contribute to organelle identity, recruit proteins important for anterograde or retrograde trafficking to the right place at the right time, and directly influence the capacity of proteins to signal to downstream partners. Our studies provide evidence in support of the importance of protein-lipid interactions in the regulation of epidermal growth factor receptor (EGFR) activity and signaling, as well as in biosynthetic trafficking from the endoplasmic recticulum (ER) to the plasma membrane (PM).

The polybasic juxtamembrane (JX) region of EGFR has been proposed to regulate protein activity by interacting with the PM and thus preventing tyrosine kinase domain activation. We demonstrate that reduction of the net charge of the JX region in wild-type EGFR results in constitutive activation of the receptor and conferral of its capacity to transform cells in a ligand-independent manner. These capabilities are maintained when the receptor is retained in the endoplasmic reticulum. In addition, we show that the polybasic JX region also plays a positive role in the response of EGFR to ligand. Receptors in which the net positive charge of the JX region is reduced show reduced activation and are deficient in downstream signaling, including their capacity to mediate Ca^{2+} mobilization.

In addition, we demonstrate that protein-lipid interactions also contribute to proper biosynthetic trafficking in cells. We provide pharmacological and molecular genetic evidence for the functional requirement of a pool of phosphoinositide 4-phosphate, synthesized by phosphoinositide 4-kinase IIIα, for ER to PM protein trafficking. In summary, strategies to perturb both protein structure and phospholipid availability provide evidence in support of roles for basic amino acid sequences and negatively charged phospholipids in biosynthetic trafficking and cellular signaling regulation.

BIOGRAPHICAL SKETCH

Kirsten was born November 5, 1985 to Bernard and Lori Elzer in Reading, Pennsylvania. She spent her formative years happily following her father around their property “helping” with yard work, as well as spending many hours at the kitchen table meticulously completing homework under the patient supervision of her mother. These hours paid off in her senior year when she graduated Valedictorian of her class at Conrad Weiser High School and was accepted for admission to Bucknell University.

At Bucknell, Kirsten majored in Biology, and in her sophomore year began serving as a teaching assistant for the Introductory Biology laboratory course. On her first day as a TA, she was assigned to work for Dr. Josef Novak. By the end of that day, Kirsten, was a member of the Novak cancer biology laboratory. She spent the next two summers as well as every semester until graduation studying the effect of serine protease treatment on neoplastic cell migration and falling in love with research.

Upon graduating Summa Cum Laude from Bucknell and receiving the Phi Sigma Award for Excellence in Research, Kirsten was accepted into the Field of Pharmacology at Cornell University as a PhD candidate and joined the laboratory of Professor Barbara Baird and Dr. David Holowka. Kirsten’s time as a PhD candidate was spent studying the epidermal growth factor receptor, in particular, a constitutively active mutant that she discovered is capable of maintaining its capacity to transform cells when retained in the endoplasmic reticulum. Aside from her academic pursuits in the Baird-Holowka laboratory, she was also the proud creator of Team Balowka, the distributor of birthday cards, and the hostess of many memorable Christmas parties.

Kirsten's biography would not be complete without mention that while at Conrad Weiser High School, she went to the Junior Prom with Matthew Bryant. They traveled to Bucknell University together, and were married while they were both PhD candidates at Cornell University. In the summer of 2013, they will leave chilly Ithaca for sunny North Carolina where Matthew has accepted a position as an Assistant Professor in the Department of Mechanical and Aerospace Engineering at North Carolina State University and Kirsten will continue her research career as a postdoctoral researcher in the laboratory of Professor Channing Der at University of North Carolina at Chapel Hill. If the last eleven years are any indication of their future, they will continue to climb, hand in hand, atop mountains, up the rungs of academia, and over the rainbow.

For my father.

"What we have to do is keep hope alive,
because without it we will sink."
-John Lennon

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First and foremost I would like to thank my advisors, Barbara Baird and David Holowka, for their consistent support and guidance throughout my time as a PhD student. One can not ask for a more inspiring mentor than Barbara Baird, and I feel like I will spend the rest of my career trying to lead more like her. My friendship with Dave is the most cherished possession that I am taking away from Ithaca with me: thank-you for always being there, to help me through the tough times as well as share in my greatest accomplishments.

Richard Cerione has gone above and beyond the typical role of a committee member. I thank him for the opportunity to spend a sabbatical in his laboratory, his willingness to participate in revising my manuscript, as well as the levity he brought to my committee meetings and exams. Thanks also to Clare Fewtrell for providing insightful guidance throughout my graduate career as the field-appointed member of my committee.

I completed my first rotation in the Cerione Laboratory, under the direction of Dr. Marc Antonyak. Marc has been a steadfast friend and mentor ever since, and I would like to thank him for that, as well as for allowing me to participate as a peripheral member of team TG throughout my time at Cornell.

As a member of the Baird-Holowka lab, I have had the pleasure of sharing my days with a number of fantastic people. Early on, Sarah Veatch was a helpful presence and great role model. Sarah Shelby's laid-back, cool brilliance has always impressed me, and while physics might always evade me, I hope to try to emulate the laid-back part. I really enjoyed collaborating with Amit on the patterned EGF project, and helping Devin take over and move that project forward has been a rewarding experience. I will always have a special place in my heart for my

fellow trafficking subgroup stepchildren: Josh Wilson, Alice Wagenknecht-Wiesner, Jinmin Lee, and Norah Smith. Norah has been a great friend who I have missed since her departure and will most likely miss even more when we are separated by far more than a mile.

While I am the first member of my family to receive a PhD, and my parents may not understand what exactly I do, they laid the groundwork for me to accomplish this goal and for that I am very grateful. I would like to thank my mom for the years she dedicated to monitoring my progress at school, and I would like to thank my dad for his steadfast confidence that I was making the right decisions and on a clear path. I would also like to thank my parents for the Kirstie Leigh and Water's Edge, the two boats we have owned while in Ithaca, which afforded me the luxury of both working and getting away on the weekends. I am also thankful to my brother, Sean, for his comedic relief when I need it.

Last but not least, I would like to thank my husband, Dr. Matthew Bryant, for walking beside me for the past eleven and a half years and for the promise of more adventures to come.

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LIST OF ABBREVIATIONS

αγγ	Chimeric receptors containing extracellular domain of FcÇRI alpha subunit, and transmembrane and cytoplasmic segments of FcÇRI gamma subunit
ARF	ADP-ribosylation factor
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
Ca^{2+}	calcium
CERT	ceramide transfer protein
DAG	diacylglycerol
DMS	N,N'-dimethylsphingosine
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant-type-three
EndoH	endoglycosidase H
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ERGIC	ER-Golgi intermediate compartment
FAPP	four phosphate adaptor protein
FFAT	two phenylalanines in an acidic track
Gab	Grb2-associated binder
GDP	guanosine diphosphate
GBM	glioblastoma multiforme
GFP	green fluorescent protein
GPI	glycophosphatidylinositol

Grb2	growth factor receptor-bound 2
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase enzyme
IgG	Immunoglobulin G
IP_3	inositol 1,4,5-trisphosphate
JX	juxtamembrane
JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
mTOR	mammalian target of rapamycin
NSCLC	non-small-cell lung carcinoma
OSBP	oxysterol-binding protein 1
PAO	phenylarsine oxide
PDI	protein disulfide isomerase
PDK1	phosphoinositide-dependent kinase 1
PI	phosphatidylinositol
PI4KIIα	alpha isoform of type II phosphatidylinositol 4-kinase
PI4KIIβ	beta isoform of type II phosphatidylinositol 4-kinase
PI4KIIIα	alpha isoform of type III phosphatidylinositol 4-kinase
PI4KIIIβ	beta isoform of type II phosphatidylinositol 4-kinase
PI4P	phosphatidylinositiol-4-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5 bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate

PKC	protein kinase C
PLC	phospholipase C
PM	plasma membrane
PNGase F	peptide-N-glycosidase F
RBL	rat basophilic leukemia
RER	rough endoplasmic reticulum
RTK	receptor tyrosine kinase
Sac1	suppressor of actin mutations 1-like protein
SER	smooth endoplasmic reticulum
SNARE	soluble NSF attachment protein receptor
Sos	son of sevenless
TGN	trans-Golgi network
TKD	tyrosine kinase domain
TMS	N,N',N"-trimethylsphingosine
TX	triton
YFP	yellow fluorescent protein

Chapter 1

Introduction

1.1 Protein-lipid interactions facilitate important cellular processes.

A central theme to what at first might seem like two very different thesis projects is the observation that proteins and lipids, specifically phosphoinositides, interact, and that these interactions serve to regulate many different cellular processes. In my first data chapter, I describe the generation of an epidermal growth factor receptor (EGFR) mutant in which the net charge of the juxtamembrane (JX) region has been reduced from +8 to +3, due to the mutation of basic residues to alanines. This receptor (Mut R1-6) is constitutively active, and we hypothesize that this is due to the release of inhibitory protein-lipid interactions that hold the receptor in an inactive conformation. In my second data chapter, I demonstrate the importance of phosphatidylinositol-4-phosphate (PI4P) in the biosynthetic trafficking of multiple classes of proteins. Biosynthetic trafficking of receptors and other transmembrane proteins from the endoplasmic reticulum (ER) to the plasma membrane (PM) underlies the capacity of these proteins to participate in crucial cellular functions, and this work adds to the body of literature describing the importance of phosphoinositides in regulating this process. In the appendix, I revisit the EGFR JX mutants and demonstrate that ablating electrostatic interactions of the JX region with the PM changes their capacity for ligand-dependent signaling as compared to wt EGFR.

The concept of protein regulation by phosphoinositides is becoming well established in the literature. Phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) serves important cellular functions as the upstream source of three second messengers. Hydrolysis of $PI(4,5)P_2$ by

phospholipase C (PLC) family members results in the generation of inositol 1,4,5-trisphosphate (IP_3) and diacyglycerol (DAG), and phosphorylation by PI3-kinase produces phosphatidylinositiol 3,4,5-trisphosphate (PIP_3). PI(4,5)P_2 also serves independently as a second messenger itself (Hurley and Meyer, 2001; Hilgemann et al., 2001). PI(4,5)P_2 has been implicated in cytoskeletal organization (Raucher et al., 2000; Yin and Janmey, 2003), membrane trafficking and exocytosis (Martin, 2001; Wenk and De Camilli, 2004), as well as in regulation of ion channel function (Logothetis et al., 2010). Furthermore, there have been several reports of PI(4,5)P_2 existing in functionally and spatially distinct pools in the PM that support specific signaling platforms (Lui et al., 1998; Johnson et al., 2008; Vasudevan et al., 2009; Calloway et al., 2011). PI(4,5)P_2 has also been shown to regulate epidermal growth factor receptor activation (Michaildis et al., 2010; see below).

PI4P, traditionally viewed as serving only as the precursor for PI(4,5)P_2, has also more recently been appreciated as an independent regulator of cellular functions. PI4P is primarily enriched in the Golgi apparatus in cells (De Matteis and D'Angelo, 2007) and thus has been implicated as a regulator of Golgi to PM secretion (Walch-Solimena and Novick, 1999; Bruns et al., 2002). PI4P has also been reported as necessary to promote COPII-mediated ER export by assisting in Sar1-induced COPII nucleation (Blumental-Perry et al., 2006).

Herein, I will review the vast EGFR literature in a way that highlights the studies most important for providing the context for my own work, beginning with a brief history of EGFR biology. I will then provide an overview of EGFR structure and the mechanism of its activation by ligand. Next, I will review mutations which result in EGFR constitutive activation, highlighting examples in which trafficking of the mutant protein is also aberrant. In the second half of the chapter, I will review the importance of phosphoinositides in regulation of cellular

signaling and biosynthetic trafficking. I will begin by highlighting the subcellular distribution of the different phosphoinositide species in cells, and then review the PI-kinases and PI-phosphatases that maintain this varied distribution. I will then focus on pharmacological and genetic methods that have been developed to interfere with this regulation. My hope is that this introduction will allow the reader to better appreciate the contribution of the following data chapters to the fields of EGFR and phosphoinositide biology, respectively.

1.2 A historical perspective on EGFR research.

The concept of signal generation from phosphorylated receptor tyrosine kinases (RTKs) that are activated by their respective ligands at the PM has been a tenet in the field of receptor biology since the early 1980's (Ushiro and Cohen, 1980). The findings that many epithelial tumors overexpress RTKs, particularly members of the ErbB family, and that they are key contributors in cancer progression (Clark et al., 1984), led to an explosion of a field that continues to grow at a rapid pace today. By the end of that decade it was understood that, like overexpression, mutations in RTKs can lead to dysregulated signaling. EGFR is the prototypic RTK.

EGF, a ligand that activates EGFR, was isolated and purified by Stanley Cohen in 1962. Within three years he furthered this discovery by showing that this molecule could stimulate the growth of epithelial cells (Cohen, 1965); these important discoveries led to his receipt of the Nobel Prize in Physiology and Medicine, which he shared with Rita Levi-Montalcini for her characterization of the related factor, nerve growth factor. It was not until 1975 that the presence of specific binding sites for EGF on the surface of cells was proposed by Graham Carpenter (Carpenter et al., 1975), a member of Cohen's lab at the time. In time they showed that this

binding site was actually a 170-kda PM-localized RTK (Carpenter et al., 1978; Ushiro and Cohen, 1980).

Throughout the 1980's, as researchers sought to better characterize the function of EGFR and other RTKs, it was routinely demonstrated that EGFR was overexpressed in a number of epithelial tumors, implicating its dysregulation in the biology of cancer (See Section 1.5). It is interesting to note that the full appreciation of EGFR signaling in normal tissues lagged behind its implication in cancer. In the mid-nineties it was demonstrated that EGFR knock-out in mice resulted in embryonic lethality or severe developmental defects in multiple organs (Threadgill et al., 1995). The abnormalities associated with EGFR deficiency confirmed that it is required for normal epithelial development.

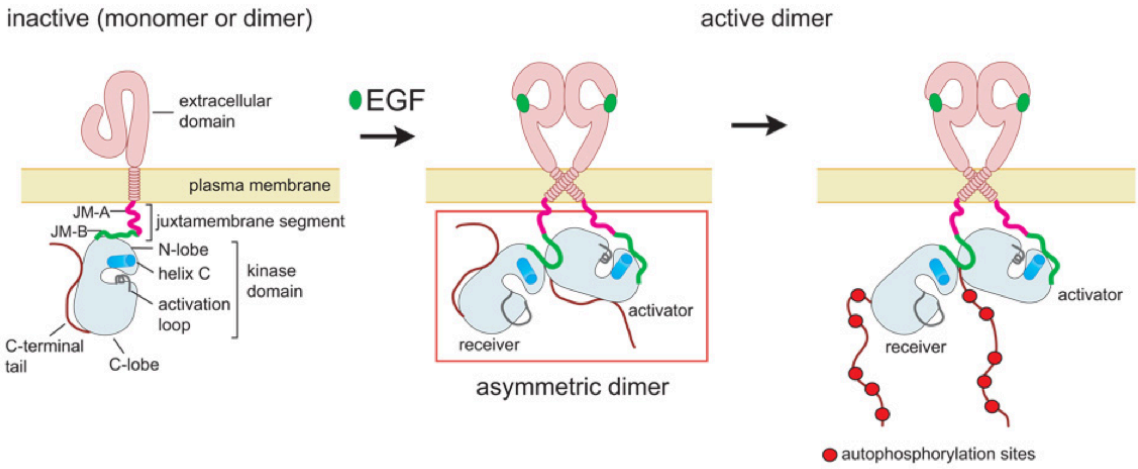
1.3 EGFR protein structure and activation.

EGFR is a Type I transmembrane glycoprotein consisting of an extracellular ligand-binding segment, a single transmembrane segment, and an intracellular segment that contains a JX region, a tyrosine kinase domain (TKD), and a C-terminal tail (Figure 1.1). While the receptor has not been crystalized in its entirety, multiple structures of pieces of the receptor with or without ligands or inhibitors have been reported. The large extracellular domains are responsible for binding to ligands including EGF, transforming growth factor α, amphiregulin, betacellulin, epigen, epiregulin, and heparin binding EGF-like growth factor (Harris et al., 2003). The extracellular domain consists of two homologous ligand-binding domains (domains I and III) as well as two cysteine-rich domains (domains II and IV). In the unliganded state, domain II participates in extensive intramolecular interactions with domain IV (Ferguson et al., 2003). Upon binding to ligand, the extracellular domain undergoes a dramatic conformational change,

through which the dimerization arm, located in domain II, is exposed and thus free to associate with domain II of the interacting receptor to form a completely receptor-mediated dimer (Figure 1.1; Ogiso et al., 2002; Garrett et al., 2002). This is in stark contrast to many other RTK dimers that rely on the ligand to provide much of the dimer interface (Hubbard and Till, 2000).

While the structure of the EGFR TKD has been extensively studied, little information is known about the presumably unstructured last ~190 amino acid C-terminal tail containing many of the phosphorylation sites important for downstream signaling (Linggia and Carpenter, 2006). The JX region of EGFR, which also lagged behind the TKD in terms of its structural characterization, has recently emerged as a key regulator of receptor function, and the current model for TKD activation can no longer be discussed without describing its involvement. The role of the JX region of EGFR in receptor activation differs from many other RTKs in that the JX region plays an activating role rather than an inhibitory role (Thiel and Carpenter, 2007).

Activation of the EGF TKD is also distinctive in that, rather than being mediated by phosphorylation, it is triggered by allosteric interactions between the TKDs as they form an asymmetric dimer in which one monomer, the activator, activates the receiver TKD (Zhang et al., 2006). The more C-terminal half of the JX region (JM-B;Figure 1.1) of the receiver TKD forms a clasp that interacts with the C-terminal portion of the activator TKD (Red Brewer et al., 2009; Jura et al., 2009). The more N-terminal half of the JX region (JM-A; Figure 1.1) stabilizes this interaction via the formation of an antiparallel helical dimer (Jura et al., 2009). TKD activation is followed by transphosphorylation of the C-terminal tails of two interacting receptors. EGFR TKDs can serve as both activators and receivers; interestingly, the kinase dead ErbB3 retains the capacity of serving as an activator TKD (Zhang et al., 2006). This observation



From: Jura et al. (2009) Cell 137(2):1293-1307.

Figure 1.1. *Activation of EGFR by EGF results in the formation of an asymmetric kinase dimer.*

mechanistically explains how ErbB3 can pair with and activate other ErbB family members (Yarden and Sliwkowski, 2001). The JX region of EGFR has also been implicated in maintaining the unliganded receptor in an inactive state. Recent evidence suggests that the basic residues in the N-terminal JX region electrostatically associate with negatively charged phospholipids to facilitate insertion of adjacent hydrophobic leucine side chains into the PM bilayer in inactive EGFR. The leucine side chains are then expelled during formation of the helical dimer in the active state (Endres et al., 2013; Arkhipov et al., 2013).

1.4 Ligand-induced EGFR downstream signaling.

The phosphorylated tyrosines on the C-terminal tail of EGFR act as docking sites for adaptor molecules that link the receptor to downstream pathways. A multitude of pathways emanate from EGFR. Herein, I will focus on the Ras/MAPK/ERK pathway, the PI3K-Akt pathway, and EGFR signaling through PLCγ (Figure 1.2).

EGFR signaling through Ras begins with the recruitment of the adaptor protein growth factor receptor-bound 2 (Grb2) to the phosphorylated receptor (Lowenstein et al., 1992). Grb2 recruits the protein son of sevenless (Sos), which acts as a guanine nucleotide exchange factor for Ras, thus transitioning it to its GTP-bound, active state (Wolfman and Marcara, 1990; Downward et al., 1990). Ras proteins communicate with more than 20 different downstream signaling partners, the most prominent of these being the Raf protein kinase family. Raf activates MEK and ERK among other signaling proteins to drive cell proliferation (Marshall, 1995; Ramos, 2008).

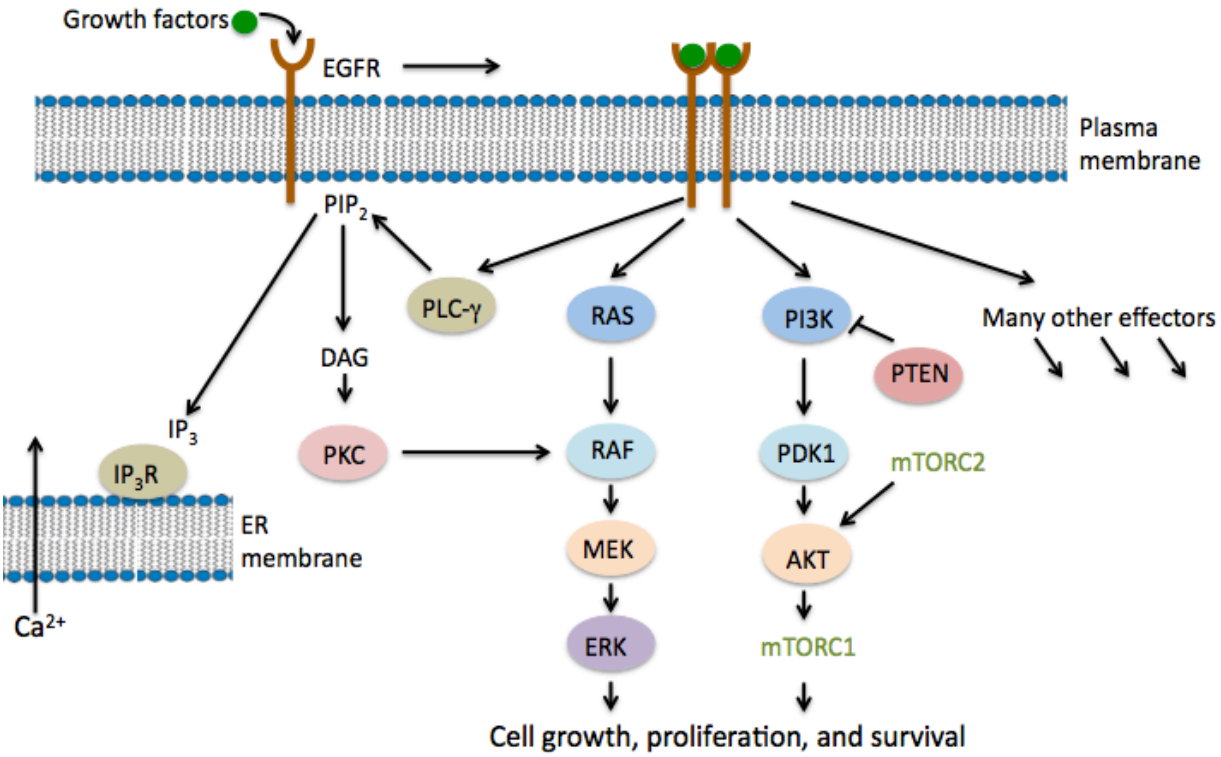


Figure 1.2. *A subset of intracellular signaling proteins influenced by EGFR.*

Phosphatidylinositol 3-kinase (PI 3-kinase) is activated by virtually all RTKs (Schlessinger, 2000). One group of PI3-kinases exists as dimers consisting of a regulatory p85 subunit and a catalytic p110 subunit. The p85 subunit is recruited to phosphorylated EGFR via the scaffold protein Grb2-associated binder (Gab) (Kong et al., 2000). Activated PI3-kinase phosphorylates $PI(4,5)P_2$ to generate $PI(3,4,5)P_3$, which in turn recruits a variety of non-receptor tyrosine kinases to the PM including PDK1 and Akt (Rameh and Cantley, 1999; Czech, 2000). Activation of Akt leads to cell survival and the evasion of apoptosis via phosphorylation and thus inactivation of the protein, BAD, which blocks its complex formation with apoptotic proteins Bcl-2 and Bcl-xl (Datta et al., 1999). Akt also phosphorylates and activates the transcription factor, FKHRI, which inhibits pro-apoptotic gene expression (Brunet et al., 1999). Furthermore, Akt can phosphorylate tuberin, which leads to activation of the mammalian target of rapamycin (mTOR) complex, a master regulator of cell growth (Manning and Cantley, 2003).

Activation of EGFR leads to phosphorylation of the receptor at tyrosine residue 992 and subsequent recruitment of PLCγ (Emlet et al., 1997). Upon phosphorylation and activation, PLCγ hydrolyzes $PI(4,5)P_2$ to form the second messengers IP_3 and DAG (Berridge, 1983). IP_3 interacts with the IP_3 receptor in the ER to mediate release of Ca^{2+} from ER stores. The released Ca^{2+} binds to calmodulin, which in turn activates Ca^{2+}/calmodulin dependent protein kinases. Meanwhile, DAG, in concert with Ca^{2+}, activates protein kinase C (PKC). PKC activation leads to phosphorylation and activation of transcription factors (Karin and Hunter, 1995).

1.5 Constitutively active EGFR mutants and their aberrant signaling.

EGFR plays important roles in cell differentiation, proliferation, and epithelial organogenesis (Carpenter, 2000). Changes in its expression and regulation, due to gene

duplication events (Xu et al., 1964; Lidermann et al., 1985; King et al., 1985; Kraus et al., 1987), in-frame deletions (Humphrey et al., 1988; Lynch et al., 2004), and point mutations (Red Brewer et al., 2009; Lynch et al., 2004) have been linked to tumor progression. Oncogenic mutations in EGFR seem to cluster in the ligand binding domain and in the TKD, two regions that are specifically important for functional regulation of the receptor.

The activity of the EGFR TKD is tightly controlled by conformational changes that occur in response to ligand binding. Mutations within this domain occur most often in non-small-cell lung carcinomas (NSCLCs) and can destabilize the inactive state of the TKD, hence leading to constitutive activation (Landau and Ben-Tal, 2008). The most common mutations that occur in this region include small deletions in the phosphate binding loop, and point mutations, such as the prototypical L858R mutation located in the activation loop (Paez et al., 2004; Greulich et al., 2005). Paradoxically, while these mutations constitutively activate the TKD, certain ones also sensitize it to inhibition with small molecules such as gefitinib or erlotinib (Carey et al., 2006; Yun et al., 2007).

As described above, formation of stable EGFR dimers follows binding of ligand to the extracellular domain of the receptor. While this process is necessary for activation, it can also be viewed as regulatory: When EGF is not bound, the receptor exists in a conformation that does not lead to signaling. The viral EGFR homologue, v-ErbB, which induces erythroblastosis in birds, completely lacks the EGF binding domain. This protein constitutively dimerizes, which leads to its constitutive kinase activation and facilitation of cellular transformation (Downward et al., 1994). A similarly mutated and constitutively active receptor, EGFR variant III (EGFRvIII), is found frequently in human glioblastoma multiforme (GBM), as well as in NSCLC, breast, and ovarian cancers (Huang et al., 1997). This mutant results from deletion of exons 2-7 of EGFR

(801 bp), which encompasses much of the ligand-binding domain. Interestingly, aside from being constitutively active, both v-ErbB and EGFRvIII have been reported to mislocalize to intracellular membranes (Privalsky and Bishop, 1984; Ekstrand et al., 1995; Gupta et al., 2010). Apart from mutants that contain large in-frame deletions, some point mutations within the ligand-binding domain have been reported to constitutively activate the receptor (Lee et al., 2006). However, these point mutations do not exhibit the trafficking defect. It is also interesting to note that while the activated wt EGFR signals through many downstream proteins (Figure 1.2), EGFRvIII exhibits downstream signaling primarily through the PI3K pathway (Moscatello et al., 1998) to JNK (Antonyak et al., 1998). The mechanism by which this particular dependence on one signaling pathway is achieved is not currently known.

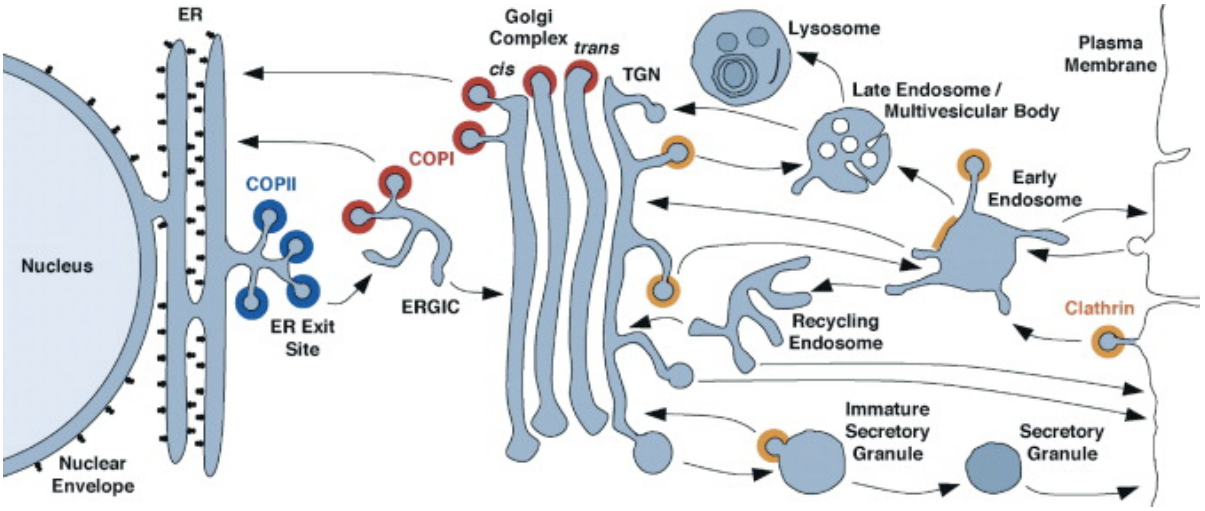
1.6 Subcellular biosynthetic trafficking is essential for delivery signaling proteins to the PM.

The secretory pathway in eukaryotic cells mediates the delivery of proteins, carbohydrates, and lipids to the cell surface. It consists of three major hubs: the ER, the Golgi apparatus, and the PM (Figure 1.3). Protein synthesis occurs at the ER, where, following synthesis proteins are packaged into COPII coated vesicles that fuse to form the ER-Golgi intermediate compartment (ERGIC) before fusing to the cis-Golgi (Klumperman et al., 1998). Following maturation through the Golgi stacks, the proteins reach the trans-Golgi network (TGN), where they are sorted for delivery to the PM. The distributions of many of these organelles were originally characterized by electron microscopy-based studies of fixed cells (Palade, 1975). Later, genetic and biochemical studies defined the machinery necessary for these events (Schekman and Orci, 1996; Rothman and Wieland 1996). More recently, studies of the

dynamics and kinetics of the process have been made more accessible with the use of live cell microscopy of fluorescently tagged proteins (Lippincott-Schwartz, 1998).

The ER is the starting point of the secretory pathway. It is composed of two subsets: the ribosome-covered rough endoplasmic reticulum (RER) responsible for protein synthesis and trafficking, and the smooth endoplasmic reticulum (SER), which is thought to be responsible for additional ER-functions such as Ca^{2+} regulation. Following insertion into the ER membrane, proteins encounter both luminal and cytoplasmic chaperone proteins; if a protein is misfolded, it is retained and degraded, and in this way the ER serves as point of quality control for the biosynthetic pathway (Hammond and Helenius, 1995). Proteins exit the ER at specialized domains called ER-exit sites, where vesicles budding from the ER are covered in a protein coat complex, COPII. COPII coat assembly begins with Sec-12 mediated GDP/GTP exchange on the GTPase Sar1 (Barlowe and Schekman, 1993). When Sar1 is bound to GTP it recruits the Sec23/24 protein complex (Yoshihisa et al., 1993), which in turn recruits the Sec13/31 complex, which polymerizes the coat to form the final COPII bud (Matsuoka et al., 1998). Upon leaving the ER, COPII vesicles are thought to fuse and become the ERGIC compartment (Klumperman et al., 1998).

When proteins reach the *cis*-Golgi apparatus, they first must pass through another round of quality control. If a protein is misfolded, it is trafficked back to the ER in COPI-coated vesicles (Pelham, 1994); COPI-mediated retrograde trafficking can occur throughout the Golgi stack. Correctly folded proteins move through the elaborate network of Golgi cisternae, which contain many different glycolipid and glycoprotein-processing enzymes. Once a protein has matured through a stack of Golgi cisternae it reaches the TGN. Exit from this structure involves sorting into distinct pathways that lead to different compartments within the cell, including the



From: Bonifacino JS and Glick BS (2004) *Cell* 116(2):153-166.

Figure 1.3. *Intracellular transport pathways.* The biosynthetic pathway is described in the text. Also, depicted here is the endosomal/lysosmal pathway in which proteins undergo retrograde transport into the cell and are either recycled back to the PM or degraded in the lysosome.

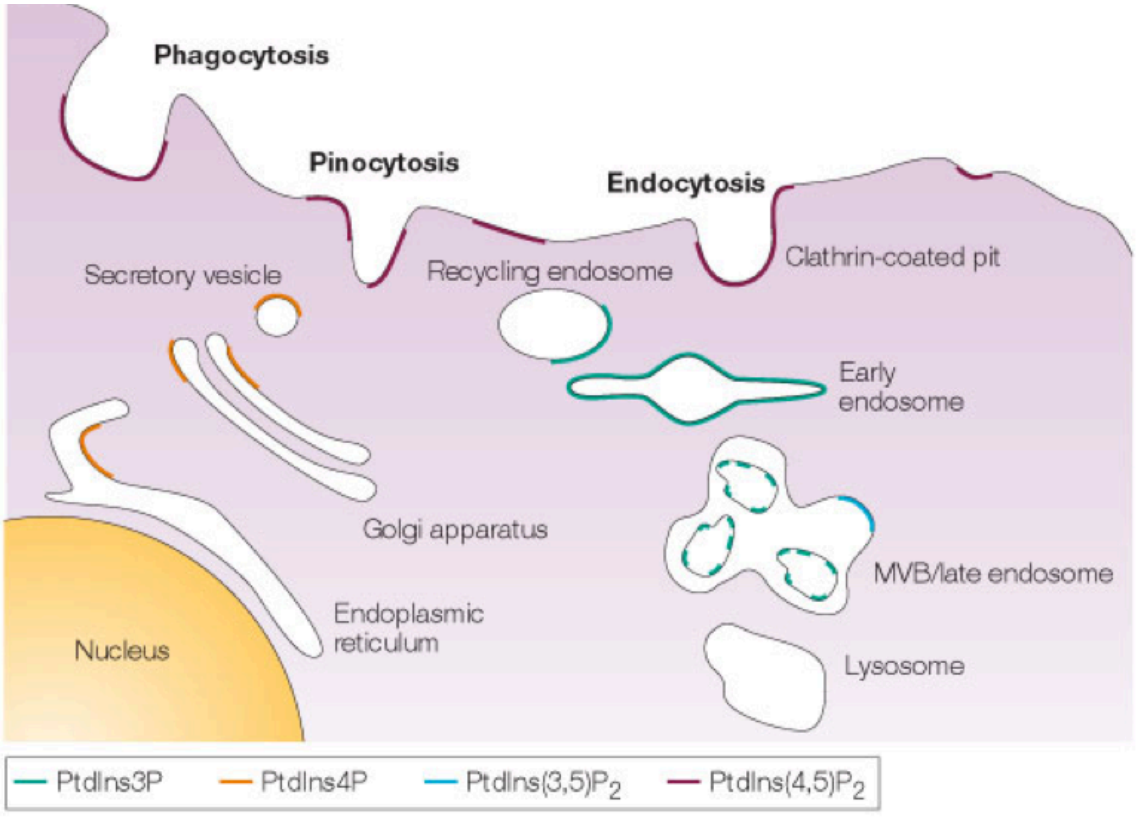
endosomal system and the PM (Keller and Simmons, 1994).

1.7 Phosphoinositides have specific subcellular distributions and regulate important cellular events, including biosynthetic trafficking.

Not addressed in the above overview of biosynthetic protein trafficking is the intricate regulation of membrane budding, trafficking, and fusion events by proteins. Coat proteins (e.g. COPI and COPII) are essential for membrane budding whereas SNARE proteins (e.g. syntaxin and synaptobrevin) regulate membrane fusion. In addition, two entire families of small GTPases, Rab- and Arf-GTPases, have evolved to regulate the process of vesicular trafficking. More recently, it has been appreciated that the phosphorylated derivatives of phosphatidylinositol (PI), or phosphoinositides, mediate the interactions of protein regulators of vesicular trafficking with target membranes.

Studies of phosphoinositides have shown that these molecules possess distinct biological functions and are localized to selective organelles (reviewed by Di Paolo and De Camilli, 2006). Due to variable phosphorylation of hydroxyl groups on their inositol rings, seven different interconvertable phosphoinositide species exist in cells, including PI4P and $PI(4,5)P_2$. Each phosphoinositide species is enriched in specific intracellular membranes (Figure 1.4); for example, $PI(4,5)P_2$ is highly localized to the inner leaflet of the PM and PI4P is enriched at the Golgi complex.

$PI(4,5)P_2$ is a well-established regulator of multiple cellular processes including vesicle trafficking and phagocytosis (Coppolino et al., 2002). Although the clathrin adaptors AP-1 and AP-2 recognize some of the same cargo proteins, the AP-2 complex is targeted to the PM due the concentration of $PI(4,5)P_2$ at this subcellular location (Honing et al., 2005). $PI(4,5)P_2$ also regulates endocytosis via interactions with dynamin (Wenk and De Camilli, 2004). Dynein



From Le Roy and Wrana (2005) Nat Rev Molec Cell Biol 6(2):112-126.

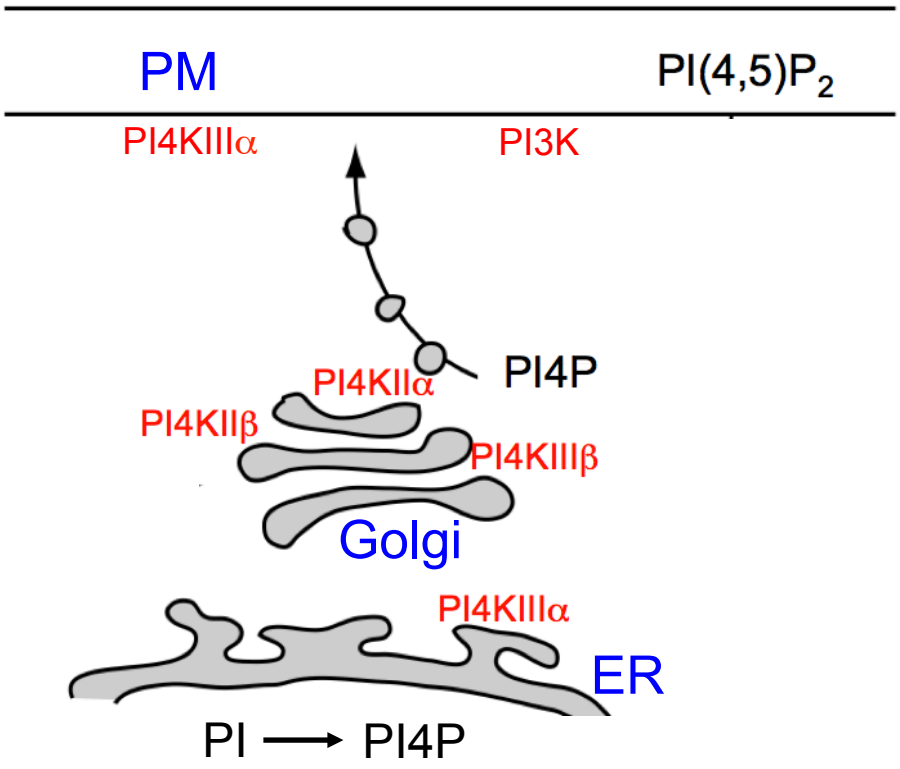
Figure 1.4. *Phosphoinositides and organelle identity.* Phosphoinositides are selectively and heterogeneously distributed throughout the cell and can be used as markers for different organelles based on their discrete subcellular distribution.

controls the membrane fission reaction, through interactions with the actin cytoskeleton, which is involved in all types of membrane endocytosis (Engqvist-Goldstein and Drubin, 2003). PI3P and $PI(3,5)P_2$ are essential for endosomal regulation, PI3P is a marker for early endosomes, and its presence is necessary for the recruitment of endosomal proteins (Birkeland and Stenmark, 2004).

PI4P is particularly important for the regulation of protein trafficking. For example, PI4P is required for COPII-mediated export of proteins at ER exit sites (Blumental-Perry et al., 2006), and, by interacting with CERT, OSBP, and FAPP lipid transfer proteins (collectively termed COFs), PI4P also plays a role in sphingolipid and sterol metabolism (D'Angelo, 2008). Also, PI4P regulates the targeting of the clathrin adaptor AP-1 complex to the Golgi, thus participating in Golgi to PM trafficking (Wang et al., 2003).

1.8 Interplay between PI 4-kinases and PI 4-phosphatases in the regulation of phosphoinositide distributions.

Organelle-specific phosphoinositide distributions are maintained by the tight regulation of cytoplasmic PI-kinases and PI-phosphatases. Four distinct PI 4-kinases have been described in mammalian cells (Figure 1.5), including type II (PI4KIIα and PI4KIIβ) and type III (PI4KIIIα and PI4KIIIβ) kinases (Balla and Balla, 2006). The type II PI 4-kinases are palmitoylated (Barylko et al., 2001) and thus strongly membrane associated, particularly in the trans-Golgi apparatus (Wang et al., 2003) and to a lesser extent in endosomes (Balla et al., 2004). PI4KIIIβ is primarily Golgi-localized (Wong et al., 1997) mostly due to its coincidence detection with Arf1, a small GTP-binding protein (Godi et al., 1999). While the molecular details of how these enzymes are linked to Golgi-derived biosynthetic transport have not been entirely elucidated,



Adapted from Szentpetery et al. (2010) PNAS, 107(18):8225-30.

Figure 1.5. *Current model of the subcellular distribution of cytoplasmic PI 4-kinases.* Because such a large amount of cellular PI4P localizes to the Golgi, some PI4P may reach the PM via vesicular transport. The primary membrane that PI4KIIIα localizes to has not been definitively established.

they have all been implicated with proper Golgi function and secretion (Balla and Ball, 2006). The localization of PI4KIIIα is not well understood. In mammalian cells, PI4KIIIα was originally reported to localize to the ER based on immunofluorescence (Wong et al., 1997). However, more recent functional studies have questioned this localization (Balla et al., 2008; Nakatsu et al., 2012) and propose that this cytoplasmic enzyme most frequently associates with the PM. The only lipid phosphatases with enzymatic activity towards PI4P are phosphoinositide phosphatases that contain Sac1 homology domains (Guo et al., 1999). The Sac1 protein is a transmembrane protein that localizes to the ER and Golgi membranes, and thus can perform organelle-specific roles in lipid signaling (Konrad et al., 2002).

The cellular functions of the different PI 4-kinases have been elucidated, in part, by the characterization of a number of different pharmacological inhibitors of these enzymes. Phenylarsine oxide (PAO) is a well-established inhibitor of PI 4-kinases (Wiedemann et al., 1996), which, when applied at very low micromolar concentrations, can selectively inhibit the PI4KIIIα isoform (Balla and Balla, 2006). Likewise, PI4KIIIβ can be selectively inhibited with PIK-93, or phenylthiazole (Knight et al., 2006). Two less isoform-specific PI4-kinase inhibitors are wortmannin and quercetin. Nanomolar concentrations of wortmannin have been shown to inhibit PI3-kinase (Arcaro and Wymann, 1993), whereas significantly higher micromolar concentrations are needed to inhibit PI4-kinases (Downing et al., 1996; Meyers and Cantley, 1997). Quercetin has been shown to generally inhibit kinases by competition with ATP for the active site (Middleton et al., 2000), and low micromolar concentrations can selectively inhibit PI-kinases (Santos et al., 2013). More recently, genetic approaches such as overexpression of PI-kinases and PI-phosphatases have been employed to modulate phosphoinositide levels in cells.

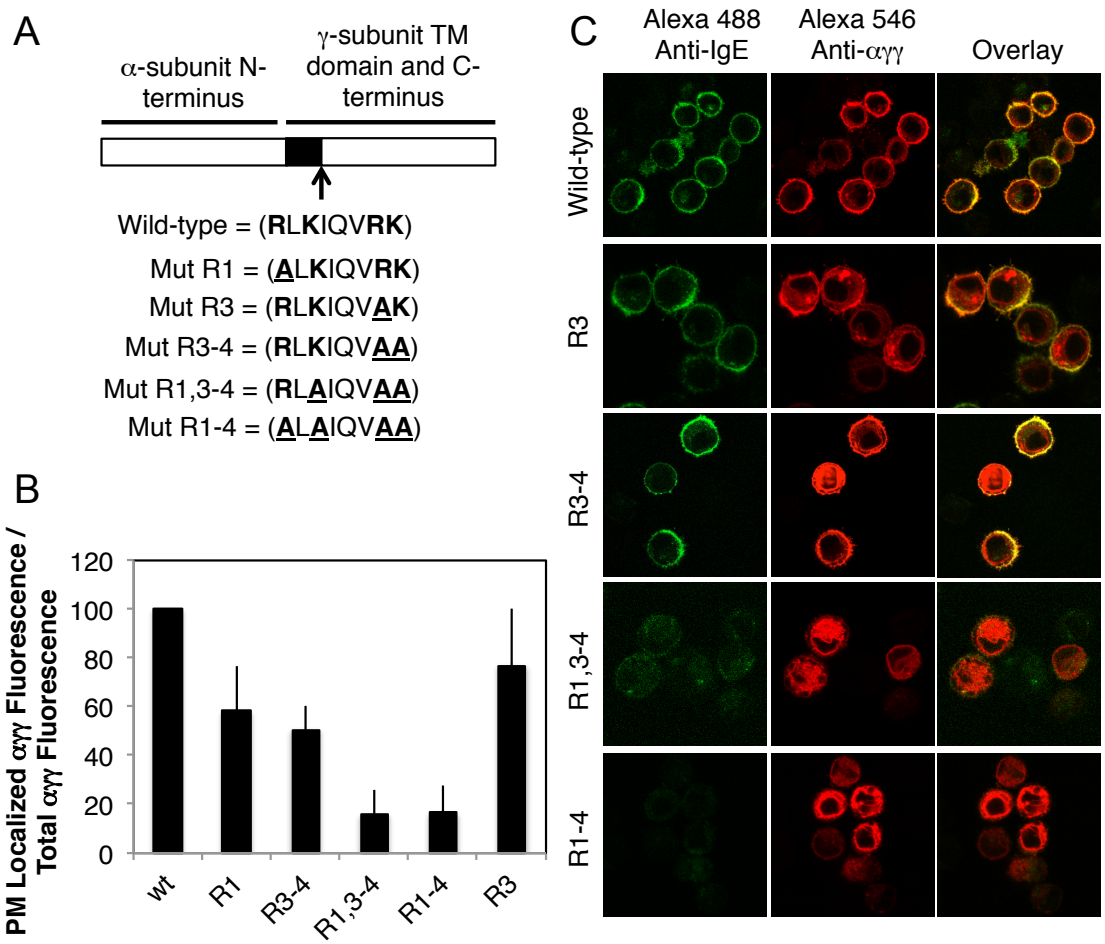
Szentpetery et al., 2010 described a useful drug-inducible molecular approach to more specifically target the effects of Sac1 phosphatase activity to a precise intracellular membrane. A similar technique has been used to deplete PIP_2 at the PM with synaptojanin (Chang-Ileto et al., 2011), and many other laboratories are modifying this technique to target different pools of phosphoinositides throughout the cell (Chang-Ileto et al., 2012).

1.9 Charge silencing mutagenesis of the polybasic JX sequence of a chimeric FcεRI receptor (αγγ) inhibits its capacity to traffic to the PM.

Our laboratory's interest in the interactions of phosphoinositides with polybasic JX regions of proteins was generated by our finding that PIP_2 positively regulates stimulated FcεRI receptor phosphorylation (Vasudevan, 2008). A candidate sequence for interactions between FcεRI and PIP_2 is the polybasic JX region of the γ-subunit of the receptor. To investigate this possibility, we utilized a single chain chimeric receptor consisting of the IgE-binding extracellular domain of the human α-subunit fused to the transmembrane and cytoplasmic domains of the γ-subunit (αγγ) previously constructed by our laboratory (Gosse et al., 2005). In the context of this chimera, we mutated the putative PIP_2-binding basic residues of the γ-cytoplasmic domain to alanines (Figure 1.6*A*).

Based on quantitative flow cytometry (Figure 1.6*B*), mutation of one or two of the basic residues caused small reductions in surface expression, but mutation of three of the four basic residues within this region completely abolished surface expression of αγγ. Rather than trafficking to the PM, the receptor was retained intracellularly, largely in the ER (Figure 1.6*C*). These results precluded a systematic investigation of the role of this polybasic sequence in FcεRI interactions with PIP_2 at the PM, but they suggested that the basic residues in the JX region of αγγ are important for its trafficking to the PM.

Figure 1.6. *Charge silencing mutagenesis of the polybasic JX sequence of the a chimeric FcεRI receptor (αγγ) inhibits its capacity to traffic to the PM.* **(A)** Wild type and mutant αγγ constructs. The JX sequence is shown: basic amino acids are in bold and mutated residues are underlined. (**B**) Quantification of the effects of mutation of the polybasic JX domain of the αγγ receptor on biosynthetic trafficking. Transiently expressing CHO cells were labeled with Alexa 488 mouse IgE for 30 min at 37°C, then fixed, permeabilized and labeled with anti-human FcεRIα Ab, followed by Alexa 546-anti rabbit IgG and analyzed using flow cytometry to determine the ratio of PM-localized A488 fluorescence to total A546-αγγ fluorescence. Data from mutants is normalized to wt, and error bars indicate ± SD of three independent experiments in which ≥3,500 cells expressing each construct were analyzed. (**C**) Confocal imaging of wt and mutant αγγ constructs in CHO cells. Transiently expressing cells were labeled with Alexa 488-mouse IgE for 30 min at 37°C, then fixed, permeabilized and labeled with rabbit anti-human FcεRIα subunit antibody, followed by Alexa546-anti-rabbit IgG.


A
α-subunit N-terminus
γ-subunit TM domain and C-terminus
Wild-type = (RLKIQVRK)
Mut R1 = (ALKIQVRK)
Mut R3 = (RLKIQVAK)
Mut R3-4 = (RLKIQVAA)
Mut R1,3-4 = (RLAIQVAA)
Mut R1-4 = (ALAIQVAA)
B
PM Localized αγγ Fluorescence / Total αγγ Fluorescence
120
100
80
60
40
20
0
wt
R1
R3-4
R1,3-4
R1-4
R3
C
Alexa 488 Anti-IgE
Alexa 546 Anti-αγγ
Overlay
Wild-type
R3
R3-4
R1,3-4
R1-4

EGFR is a Type I transmembrane protein that contains a polybasic JX domain analogous to that of FcεRIγ (Sato et al., 2006). Our studies with EGFR began when we performed similar mutagenesis on this receptor to test the generality of our hypothesis that such basic residues are important for ER to PM trafficking. Following the line of evidence that originally led to our mutation of the polybasic JX region of EGFR, one can appreciate that the observed ER-retention that occurred when six of the nine basic residues were mutated to alanines (Figure 2.1), was originally viewed as support of our αγγ data. Our guiding hypothesis at that time, that electrostatic interactions between the basic regions of proteins and acidic phospholipids were important for proper biosynthetic trafficking, is what led us to study both the role of the polybasic JX region of EGFR in its signaling (Chapter 2) and the importance of PI4P in ER to PM trafficking (Chapter 3).

1.10 Contributions

The juxtamembrane region of EGFR has recently emerged as a key regulator of receptor function (Red Brewer et al., 2009; Jura et al., 2009; Arkhipov et al., 2013). In Chapter 2 of this dissertation, I demonstrate that reduction of the net charge in the JX region of wild-type EGFR results in constitutive activation of the receptor. Furthermore, I show that this activity is maintained when the receptor is retained in the ER via a novel point mutation that we describe. Importantly, this ER-retained mutant EGFR exhibits constitutive auto-phosphorylation in the absence of ligand binding and is capable of inducing cellular transformation by preferentially signaling through PI3K and mTOR. In addition, I show that the highly oncogenic mutant EGFRvIII is wholly retained in the ER of U87 glioblastoma cells and is robustly phosphorylated at this intracellular membrane, demonstrating that this EGFR deletion mutant, implicated in

human brain cancers, can be oncogenic in an intracellular location. Taken together, these findings provide strong evidence that constitutively active EGFRs are capable of sending transforming signals from the ER.

In Chapter 3, I further explore the range of ways that phospholipids can regulate protein dynamics, in this case, in terms of proper biosynthetic trafficking. The described results support the notion that a pool of PI4P synthesized by PI4KIIIα is functionally required for proper ER-to-Golgi trafficking. Using a novel technique that I designed to monitor biosynthetic trafficking, I demonstrate that pharmacological inhibition of PI4KIIIα results in ER-retention of both the epidermal growth factor receptor (EGFR) and a GPI-anchored protein. Furthermore, I demonstrate that specific inhibition of a Golgi-localized PI 4-kinase does not result in ER-retention. Finally, I show that depletion of PI4P via overexpression of the PI-phosphatase Sac1 similarly inhibits protein trafficking to the PM. Taken together, these findings provide evidence that a specific pool of PI4P, synthesized by PI4KIIIα, is essential for proper biosynthetic protein trafficking.

In Appendix A, I present very recent data assessing the ligand-induced signaling capabilities of EGFR JX mutants localized to the PM. These receptors do not generate the same magnitude of a Ca^{2+} response as wt EGFR, and I provide evidence that this can be explained by decreased activation of downstream signaling partners such as PLCγ-1. I hypothesize that this phenomenon is due to the constitutive signaling of JX mutant receptors and thus the lack of an un-phosphorylated pool of downstream signaling partners primed for activation.

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Chapter 2

Ligand-independent Cell Transformation by an ER-retained EGF Receptor Mutant

2.1 Abstract

Deregulation of ErbB receptor tyrosine kinases is a hallmark of many human cancers. Conserved in the ErbB family is a cluster of basic amino acid residues in the juxtamembrane (JX) region. We found that charge-silencing mutagenesis within the JX region of the epidermal growth factor receptor (EGFR) results in the generation of a mutant receptor (EGFR R1-6) that spontaneously transforms NIH 3T3 cells in a ligand-independent manner. The capacity of EGFR R1-6 to mediate ligand-independent transformation is maintained when this mutant is retained in the endoplasmic reticulum (ER) via a single point mutation, L417H, which we describe. We show that EGFR R1-6 with L417H exhibits basal tyrosine phosphorylation when ectopically expressed, and its transforming activity is both sensitive to inhibition of EGFR kinase activity and particularly dependent on PI3-kinase and mTOR activity. We also evaluate EGFR variant type III (EGFRvIII), which is a constitutively active, highly oncogenic mutant form of EGFR that has been linked to human brain cancers, although how it transforms cells has not been clearly defined. We show that, similar to EGFR R1-6/L417H, EGFRvIII confers transforming activity while it is wholly ER-retained in U87 cells. These findings demonstrate that ER-localized EGFR mutants are capable of potent transforming activity.

2.2 Introduction

Receptor tyrosine kinases (RTKs) initiate some of the most complex signaling networks in cells (Lemmon and Schlessinger, 2010). To maintain normal cellular homeostasis, RTK signaling must be tightly regulated. Loss or perturbation of this regulation leads to defects in cellular processes that give rise to oncogenic transformation (Blume-Jensen and Hunter, 2001). Oncogenic mutations in RTKs can lead to their over-expression, constitutive activation, and/or mislocalization in cells.

The ErbB family and, in particular, ErbB1, or the epidermal growth factor receptor (EGFR), serves as a prototypic model for RTK signaling. EGFR is a Type I transmembrane glycoprotein consisting of an extracellular ligand-binding segment, a single transmembrane segment, and an intracellular segment that contains a JX region, a tyrosine kinase domain (TKD), and a C-terminal tail. Signaling by EGFR is initiated following its ligand-induced dimerization, which leads to TKD-dependent phosphorylation of the C-terminal segments of two interacting receptors (Schlessinger, 2000). The ~37-residue JX region of EGFR has emerged as a key regulator of receptor function (Red Brewer et al., 2009; Jura et al., 2009; Arkhipov et al., 2013). Several intrinsic sorting signals have been mapped to this JX region (Morrison et al., 1996; He et al., 2002; Hsu and Hung, 2007), which also contains a basic calmodulin-binding sequence (Martin-Nieto and Villaobo, 1998) that additionally interacts with acidic phospholipids (McLaughlin et al., 2005; Sengupta et al., 2009; Michailidis et al., 2011). In an active receptor dimer, the C-terminal halves of the JX region have been shown to clasp two interacting EGFRs together in a conformation that promotes their activation (Red Brewer et al., 2009; Jura et al., 2009), whereas the N-terminal halves of these domains stabilize this interaction via the formation of an antiparallel helical dimer (Jura et al., 2009). Recent evidence suggests that basic residues in

the N-terminal JX region facilitate insertion of adjacent hydrophobic amino acid side chains into the plasma membrane (PM) bilayer in inactive EGFR, and these leucine side chains are expelled during formation of the helical dimer in the active state (Arkhipov et al, 2013, Endres et al., 2013).

EGFR plays important roles in cell differentiation, proliferation, and epithelial organogenesis (Carpenter, 2000). Changes in its expression and regulation, due to gene duplication events (Xu et al., 1984; Libermann et al., 1985; King et al., 1985; Kraus et al., 1987), in-frame deletions (Humphrey et al., 1988; Lynch et al., 2004), and point mutations (Lynch et al., 2004; Red Brewer et al., 2009) have been linked to tumor progression. EGFRvIII (for EGFR variant type III) is an example of an in-frame deletion of EGFR found frequently in glioblastoma multiforme (GBM), as well as in non-small cell lung carcinomas, breast, and ovarian cancers. Expression of this tumor-specific mutant receptor occurs in one-third of GBMs and is an independent negative prognostic indicator of survival (Heimberger et al., 2005). It results from deletion of exons 2-7 of EGFR (801 bp), which encompasses much of the ligand-binding domain. This mutant exhibits constitutive phosphorylation (Huang et al., 1997) and downstream signaling through the PI3K pathway (Moscatello et al., 1998) to JNK (Antonyak et al., 1998). While it has been reported that EGFRvIII is completely (Eckstrand et al., 1995) or partially (Gupta et al., 2010) mislocalized intracellularly, this remains an area of controversy (Wikstrand et al., 1997; Johns et al., 2005; Cvrljevic et al., 2011). The critical question we address in the present study is whether a constitutively active variant EGFR that is fully retained in the ER can cause cellular transformation.

Motivated by the hypothesis that electrostatic binding of the polybasic JX region of EGFR to acidic phospholipids restricts access of the kinase domain to substrate tyrosines, we

demonstrate that reduction of the net charge in the JX region of wild-type (wt) EGFR results in constitutive activation of the receptor. Furthermore, we show that this activity is maintained when the receptor is retained in the ER via a novel point mutation that we describe. Importantly, we find that this ER-retained mutant EGFR exhibits constitutive auto-phosphorylation in the absence of ligand binding and is capable of inducing cellular transformation by preferentially signaling through PI3K and mTOR. In addition, we show that the highly oncogenic mutant EGFRvIII is wholly retained in the ER of U87 glioblastoma cells and is robustly phosphorylated at this intracellular membrane, demonstrating that this EGFR deletion mutant, implicated in human brain cancers, can be oncogenic in an intracellular location. Taken together, these findings provide strong evidence that constitutively active EGFRs are capable of sending transforming signals from the ER.

2.3 Materials and Methods

2.3.1 Materials

All cell culture reagents, EGF, and precast gels for blotting were from Invitrogen. FuGene HD was from Roche Applied Sciences. The anti-N-terminal (clone LA1) and anti-C-terminal (clone E235) human EGFR antibodies (used for immunochemistry), as well as the PVDF filters, were from Millipore Corp. The anti-PDI mAb was from Affinity Bioreagents. All Alexa dye-conjugated secondary antibodies were from Invitrogen, and the Cy5-conjugated anti-rabbit IgG was from Jackson ImmunoResearch Laboratories. The antibodies that recognize the phosphorylated forms of Akt and Erk, as well as phospho-specific EGFR antibody (1068), the anti-EGFR (used for blotting), and anti-phosphotyrosine antibodies were from Cell Signaling.

The anti-actin antibody was from LabVision/Thermo. HRP-conjugated secondary antibodies used for blotting were from GE Healthcare. LY294002, PD98059, and rapamycin were from EMDBiosciences. Gefitinib (Iressa) was from Selleck Chemicals. The Endo H and PNGase F, as well as restriction enzymes (DpnI, XbaI, EcoRI, MfeI), and the Phusion High-Fidelity DNA Polymerase were from New England Biolabs. The anti-GFP mAb, monoclonal anti-EGFR (clone 225, Cetuximab), as well as any chemical not noted otherwise were purchased from Sigma-Aldrich Chemical Co.

2.3.2 Expression Plasmids

The human EGFR-GFP construct has been described previously (Monick et al., 2005). To generate the juxtamembrane mutants, site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) or Phusion High-Fidelity DNA Polymerase (New England BioLabs). Amino acid numbering includes the 24-residue membrane-targeting signal sequence. Prior to the generation of stable cell lines, the wt and mutant EGFR constructs were cloned into the pKH3 expression vector (Addgene) using the Xba1 and EcoR1 restriction sites. Primers containing the XbaI and MfeI restriction sites were annealed to the N and C-termini, respectively, of EGFR constructs, due to the presence of an EcoRI restriction site within EGFR. The particular restriction sites were chosen to eliminate the triple-HA tag, present in the pkH3 vector, from the final protein product. EGFR-vIII cDNA was obtained in an MSCV-XZ066 vector (Addgene). The EGFR-vIII-pkH3 construct was made as described above for the other EGFR-constructs.

2.3.3 Cell Culture

Rat RBL-2H3 mast cells were grown in MEM containing 20% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals) and 10 μg/ml gentamicin sulfate as described previously (Gosse,

2005). In preparation for imaging and flow cytometry, cells were plated at 25-50% confluency into 35 mm MatTek wells (MatTek Corporation) or 60 mm dishes, respectively. After approximately 20 h, RBL-2H3 cells were transfected with either mutant or wild-type versions of EGFR. These constructs were transfected using Fugene HD (Roche) per manufacturers' instructions, with modification to enhance transfection efficiency in the RBL cells previously described (Gosse et al., 2005). Cells were processed for imaging or flow cytometry 24 h after transfection.

Human HeLa cells were grown in RPMI containing 10% FBS. Mouse NIH 3T3 cells were grown in DMEM containing 10% (v/v) calf serum (CS). In preparation for imaging, cells were plated at 50% confluency into 35 mm MatTek wells. After approximately 20 h, cells were transfected with either mutant or wild-type versions of EGFR. These constructs were transfected using Fugene HD (Roche) per manufacturers' instructions. U87 cells were grown in DMEM supplemented with 10% (v/v) FBS. Where indicated, NIH 3T3 or U87 cells were treated with 0.1 μg/ml or 0.02 μg/ml EGF. To generate stable cell lines, NIH 3T3 or U87 cells were transfected with the various pkH3 vectors, either alone or incorporating EGFR constructs, using FuGene HD (Roche). Transfected cells were maintained in DMEM supplemented with 10% CS (NIH 3T3) or 10% FBS (U87) and 2 μg/ml puromycin (Invitrogen). After 10–14 days, puromycin-resistant colonies were selected (NIH 3T3) or pooled (U87) and subcultured in DMEM supplemented with 10% CS (NIH 3T3) or 10% FBS (U87) and 0.5 μg/ml puromycin. The clones/lines were then assessed for expression of each respective EGFR construct.

2.3.4 Confocal Fluorescence Microscopy

NIH 3T3 and RBL-2H3 cells were washed once with BSS (BSS: 20 mM HEPES, 135 mM NaCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml BSA, pH 7.4), fixed using

4% paraformaldehyde with 0.1% glutaraldehyde, permeabilized (or not) with either 1% v/v Triton X-100 or 0.05% w/v saponin, and labeled for 1 hour with specified antibodies in phosphate buffered saline (PBS) with 10 mg/ml BSA and 0.01% w/v sodium azide (PBS/BSA). Images were collected using an upright Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Exton, PA) with a 63 x 0.9 NA, HCX APO L U-V-I water-immersion objective.

2.3.5 Flow Cytometry

RBL-2H3 cells were harvested, washed once with BSS, fixed using 4% paraformaldehyde with 0.1% glutaraldehyde, and washed once with PBS/BSA. Cells expressing GFP-tagged wild-type or mutant EGFR constructs were labeled with appropriate antibodies for 1 h in PBS/BSA. Samples were evaluated using a Becton Dickinson LSR II flow cytometer, and data were analyzed using BD FACSDiva software. Analysis was gated to include single cells on the basis of forward and side light-scatter and data from single-color samples were used to determine the gates for positive fluorescence from each fluorophore.

2.3.6 Immunoblot Analysis

Cells were washed in PBS, incubated in lysis buffer (25mM Tris, pH7.4, 100mM NaCl, 1mM EDTA, 1% (v/v) Triton 100, 1mM DTT, 1mM sodium orthovanadate, 1mM β-glycerol phosphate, 1μg/mL leupeptin, and 1μg/mL aprotinin), and supernatants were retained following microfuge centrifugation. Protein concentrations of the whole-cell lysates (WCL) were determined using the Bio-Rad DC protein assay. The WCL (35 μg/lane) were resolved by SDS/PAGE, and the proteins were transferred to PVDF membranes. The filters were blocked in 10% BSA diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 and then incubated with the indicated primary antibodies diluted in the same buffer. The primary antibodies were

detected with HRP-conjugated secondary antibodies followed by exposure to ECL reagent (Invitrogen).

2.3.7 Endo H Sensitivity Assay

NIH 3T3 or U87 cells were lysed as above, and WCLs were divided into three equal portions, which then were not treated or treated with endoglycosidase H (Endo H) (Maley et al., 1989) or peptide: N-glycosidase F (PNGase F) according to the manufacturer's instructions (New England Biolabs). Samples were incubated for 24 hours at 37°C, and the digestion reactions were stopped by boiling at 95°C for 5 min after the addition of SDS-PAGE sample buffer. Samples were analyzed by immunoblotting.

2.3.8 Anchorage-independent Growth Assays

NIH 3T3 cells stably overexpressing vector only (control), wt EGFR, EGFR Mut R1-5, EGFR Mut R1-6, EGFR L417H, or EGFR R1-6/L417H were plated at densities of 8.5 x 10^3 cells/ml in medium containing 0.3% agarose, with or without EGF or specified inhibitors, onto underlays composed of growth medium containing 0.6% agarose in six-well dishes. The soft agar cultures were re-fed with fresh medium (including EGF or specified inhibitors) every fifth day for 15-20 days, at which time the colonies that had formed were counted. Each of the assays was performed at least three times, and the results were averaged and expressed +/- SD. Experiments using parental U87 cells and cells stably expressing EGFRvIII were performed similarly except 3 x10^3 cell/ml were plated in medium containing 0.3% agarose and colonies were enumerated after 6 days, including re-feeding with fresh medium inhibitor on day 3.

2.3.9 Data Analysis

Statistical analyses were determined using GraphPad Prism (GraphPad Software, La Jolla, CA), using the Student *t* test, with $p \leq 0.05$ considered statistically significant.

2.4 Results

2.4.1 A single point mutation within the N-terminal domain of EGFR prevents its trafficking to the plasma membrane.

In an effort to investigate the potential roles of basic JX residues in receptor-mediated cell signaling, we focused on the polybasic JX region (Figure 1*A*) of EGFR (Aifa et al., 2006; Sato et al., 2006; Jura et al., 2009; Theil and Carpenter, 2007). Based on the work of others (McLaughlin et al., 2005; Sengupta et al., 2009), we hypothesized that charge silencing within the JX sequence of EGFR would release regulatory electrostatic interactions with membrane lipids and thereby promote the activity of the TKD (see Discussion). We sequentially mutated basic residues in this sequence to alanines within a C-terminal GFP-tagged EGFR (Figure 2.1*A*). Whereas wt EGFR-GFP is found almost exclusively at the PM when expressed in RBL mast cells, reduction in the net charge of the JX region from +8 to +3 (EGFR Mut R1-6) initially appeared to result in complete intracellular retention. However, sequencing revealed that unintentional mutagenesis occurred during the design of Mut R1-6, and that a single point mutation, L417H, in the EGFR extracellular domain is responsible for the trafficking defect (EGFR Mut R1-6/L417H). When this single point mutation was made in the context of wt EGFR (wt EGFR L417H), trafficking was similarly prevented, as evidenced by both confocal imaging (Figure 2.1*B*) and quantitative flow cytometry (Figure 2.1*C*).

Figure 2.1. *A single point mutation L417H within the N-terminal segment of EGFR inhibits its capacity to traffic to the plasma membrane.* (**A**) Wild-type and mutant EGFR-GFP constructs. The JX sequence in EGFR is shown, with basic amino acids in bold and mutated residues underlined. (TM = transmembrane) (**B**) Expression of wt and mutant EGFR-GFP constructs in suspended RBL mast cells. Transiently-expressing cells were fixed and labeled with an anti-N-terminal EGFR antibody, followed by Alexa647-anti-IgG. (**C**) Quantification of the effect of mutation of the polybasic JX segment of EGFR and the L417H point mutation (LH) on biosynthetic trafficking. RBL-2H3 cells transiently expressing GFP-tagged EGFR constructs were harvested, fixed and labeled with anti-N-terminal EGFR antibody followed by Alexa647-anti-IgG, then analyzed by flow cytometry to determine the ratio of PM-localized EGFR fluorescence to total-EGFR fluorescence. Data from mutants are normalized to wt and error bars indicate ± SD of three independent experiments in which ≥8,000 cells expressing each construct were analyzed.

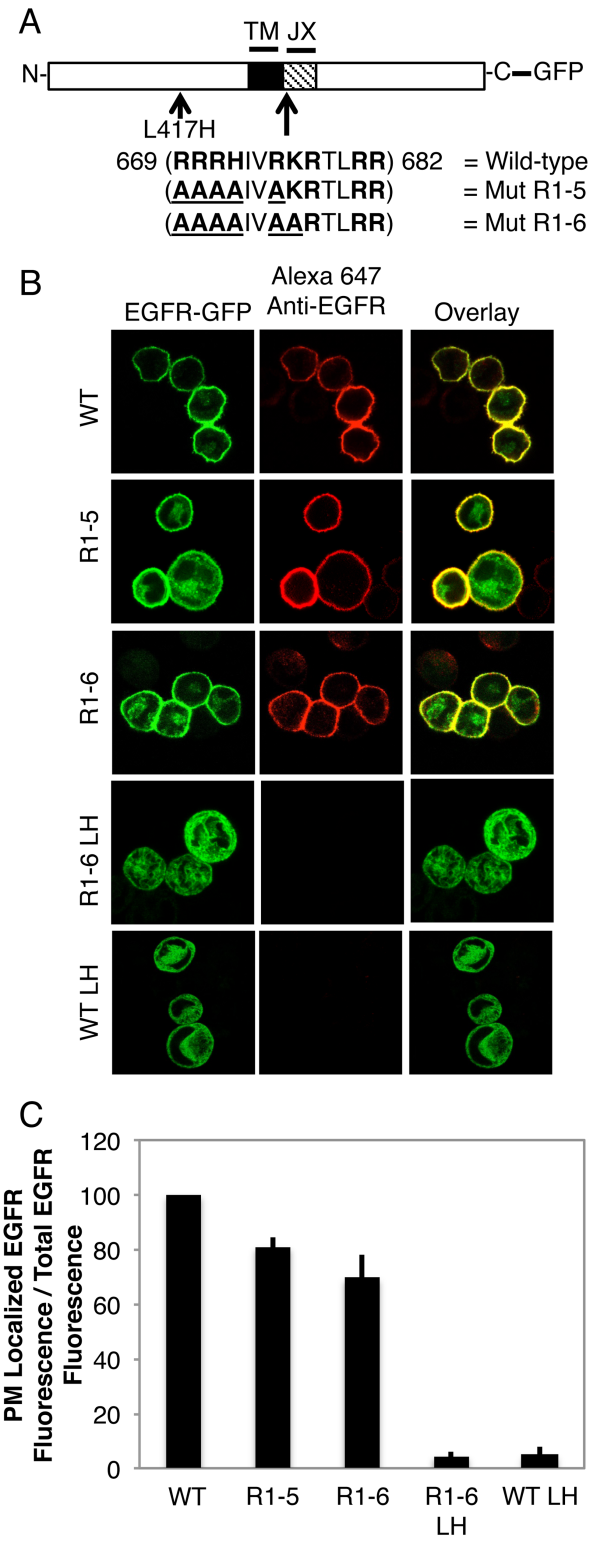
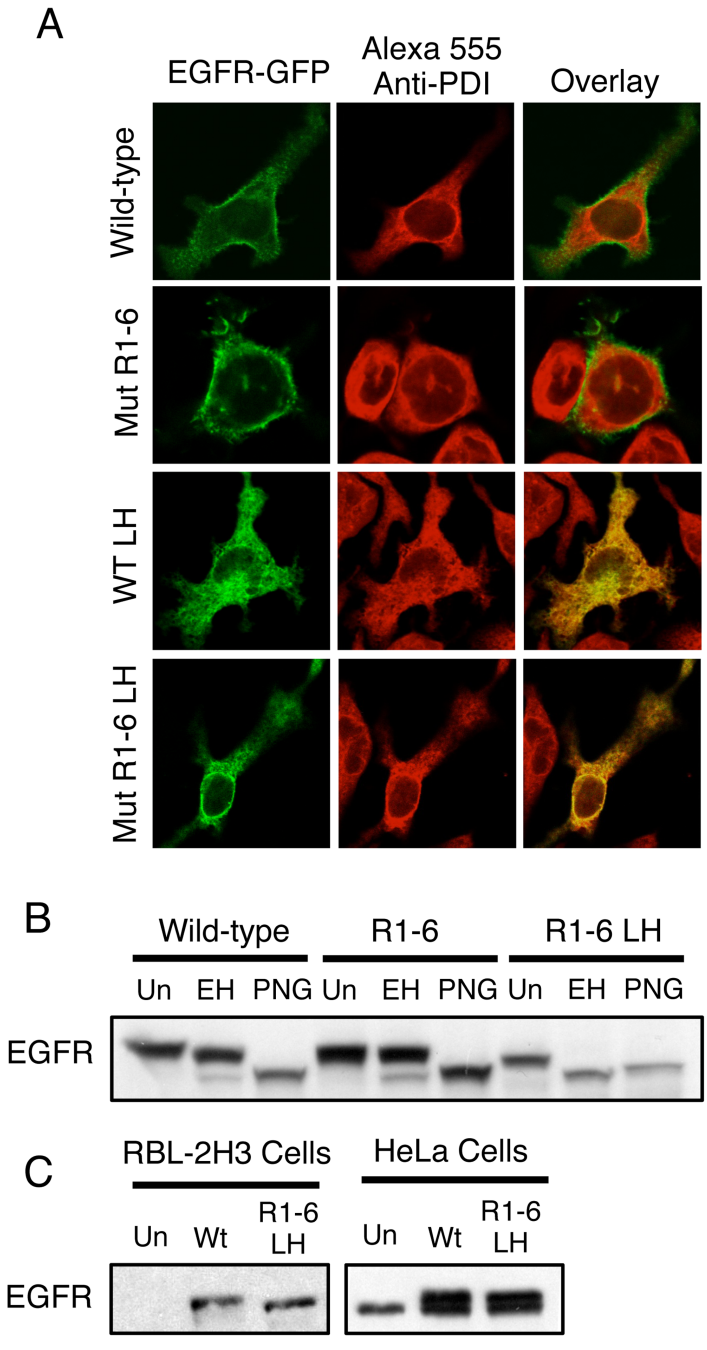

A
TM JX
N-
-C—GFP
L417H
669 (RRRHIVRKRTLRR) 682 = Wild-type
(AAAAIVAKRTLRR) = Mut R1-5
(AAAAIVAARTLRR) = Mut R1-6
B
EGFR-GFP
Alexa 647
Anti-EGFR
Overlay
WT
R1-5
R1-6
R1-6 LH
WT LH
C
PM Localized EGFR
Fluorescence / Total EGFR
Fluorescence
120
100
80
60
40
20
0
WT
R1-5
R1-6
R1-6 LH
WT LH

Figure 2.2. *EGFR-GFP Mut R1-6/L417H is retained and intact within the ER.* (**A**) Attached RBL mast cells transiently expressing wt EGFR-GFP, EGFR-GFP Mut R1-6, EGFR-GFP L417H, or EGFR-GFP Mut R1-6/L417H were fixed, permeabilized, and labeled with an anti-PDI antibody followed by Alexa555-anti-mouse IgG to label the ER. (**B**) Whole cell lysates from RBL mast cells transiently expressing either wt EGFR, EGFR Mut R1-6, or EGFR Mut R1-6/L417H were untreated (Un), treated with Endo H (EH), or treated with PNGase F (PNG) for 24 hours before Western blot analysis with an anti-EGFR antibody. (**C**) Whole cell lysates of either RBL-2H3 or HeLa cells untransfected (Un) or transiently expressing either wt EGFR (Wt) or EGFR Mut R1-6/L417H (R1-6 LH) were probed with an anti-EGFR antibody. Lower band in doublet bands is endogenous EGFR.

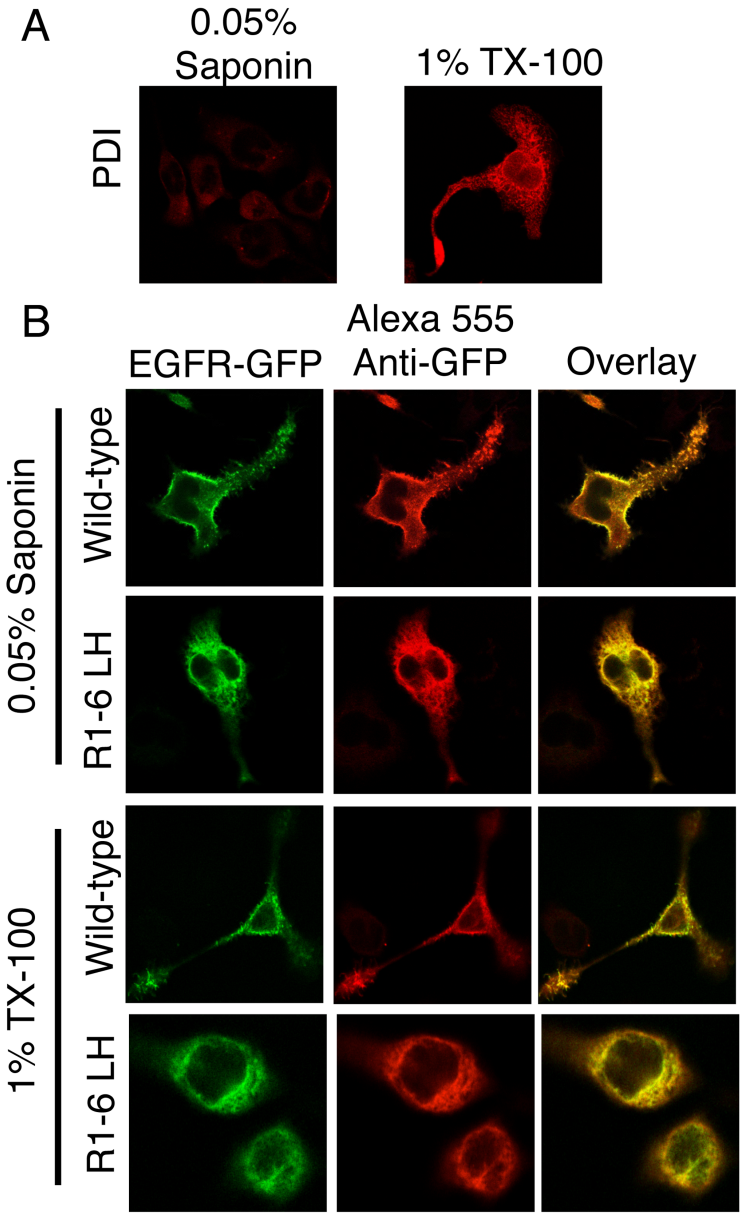

A
EGFR-GFP
Alexa 555
Anti-PDI
Overlay
Wild-type
Mut R1-6
WT LH
Mut R1-6 LH
B
Wild-type
R1-6
R1-6 LH
Un EH PNG Un EH PNG Un EH PNG
EGFR
C
RBL-2H3 Cells
HeLa Cells
Un Wt R1-6 LH
Un Wt R1-6 LH
EGFR

Strong evidence that EGFR L417H and EGFR Mut R1-6/L417H localize to the ER is provided by immunofluorescent colocalization with the resident lumenal ER protein, PDI (Figure 2.2*A*), and by complete sensitivity of EGFR MutR1-6/L417H to digestion by endoglycosidase H (Endo H; Figure 2.2*B*). The Endo H sensitive band is the same molecular weight as the Peptide-N-Glycosidase F (PNGase F)-sensitive band. PNGase F removes all glycosylations, and serves as a control for these assays. In contrast, wt EGFR and EGFR Mut R1-6 show only a small amount of Endo H sensitivity, consistent with confocal imaging and flow cytometry results showing predominant surface localization for both of these proteins (Figure 2.1). Immunoblot analysis shows that EGFR Mut R1-6/L417H retained in the ER is intact both in RBL-2H3 cells, which do not endogenously express EGFR, and in HeLa cells, which have high levels of endogenous EGFR (Figure 2.2*C*). ER-retained EGFR Mut R1-6/L417H appears to be oriented correctly across the ER membrane, with its C-terminal domain extending into the cytosol: Confocal imaging of cells in which the PM (but not the ER) was selectively permeabilized with a low concentration of saponin showed that these receptors are labeled with anti-GFP antibody (Figure 2.3). Collectively, these results show that EGFR Mut R1-6/L417H is confined to the ER, whereas EGFR Mut R1-6 is largely expressed at the PM.

2.4.2 ER-retained EGFR Mut R1-6/L417H transforms cells independently of EGF.

We hypothesized that charge silencing within the JX sequence of EGFR would release regulatory electrostatic interactions with acidic phospholipids and thereby promote the activity of the TKD. Thus, we tested the possibility that EGFR Mut R1-6 would promote cell transformation due to constitutive activation by generating NIH 3T3 cell lines stably over-

Figure 2.3. *EGFR-GFP Mut R1-6/L417H is correctly oriented across the ER membrane.* (**A**) The lumen of the ER, marked by PDI, is accessed when 1% TX-100 is used as a permeabilization agent, but not with 0.05% saponin, which permeabilizes the PM without permeabilizing internal membranes. RBL-2H3 cells were fixed, permeabilized as indicated, and labeled with an anti-PDI mAb followed by Alexa 555-anti-mouse IgG. (**B**) RBL-2H3 cells transiently expressing wt EGFR-GFP or EGFR-GFP Mut R1-6/L417H were fixed, permeabilized as indicated and labeled with anti-GFP mAb followed by Alexa555-anti-mouse IgG to label the C-terminus of the expressed EGFR construct. The GFP epitope on the EGFR-GFP Mut R1-6/L417H C-terminus is labeled following permeabilization with either 0.05% saponin or TX-100, consistent with cytoplasmic exposure.

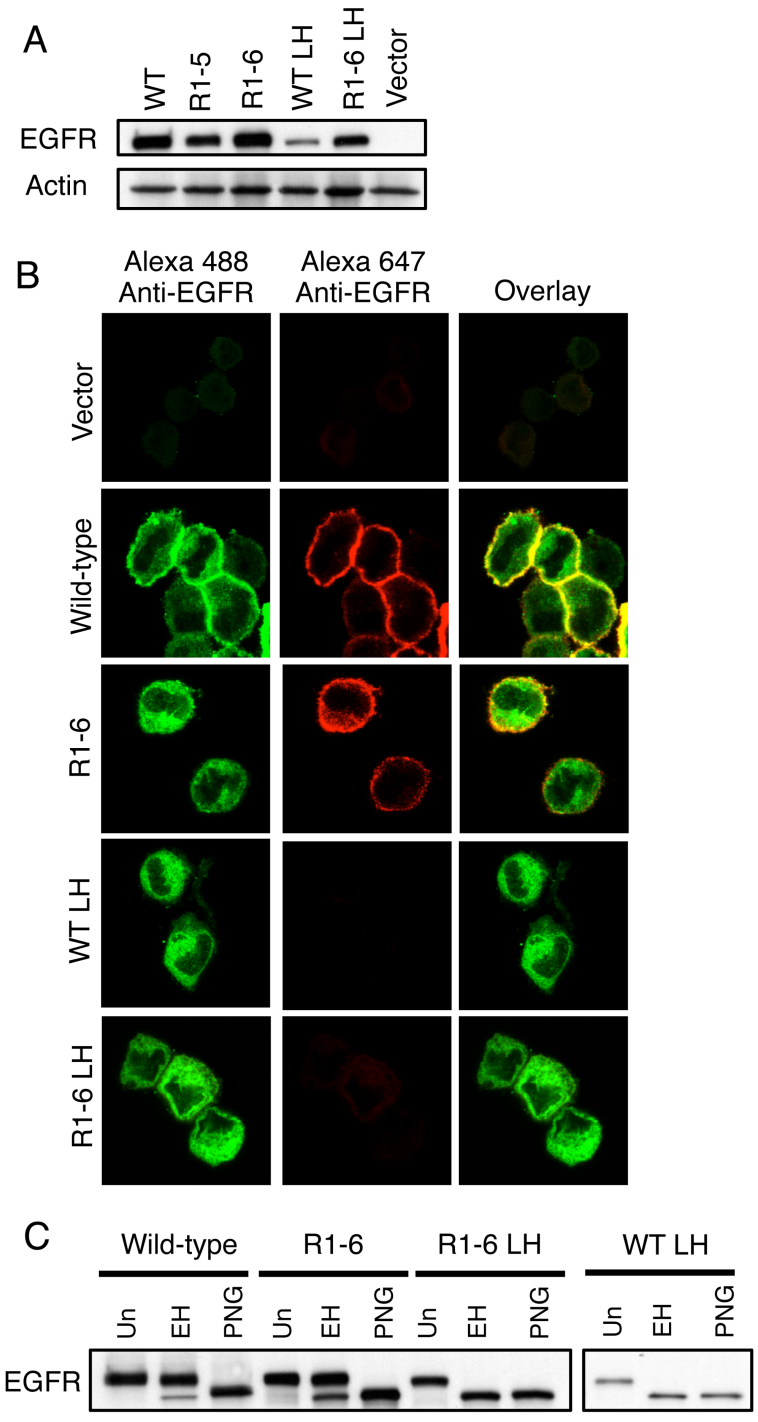

A
0.05%
Saponin
1% TX-100
PDI
B
Alexa 555
EGFR-GFP
Anti-GFP
Overlay
0.05% Saponin
Wild-type
R1-6 LH
1% TX-100
Wild-type
R1-6 LH

expressing wt EGFR, EGFR Mut R1-5, EGFR Mut R1-6, EGFR L417H, and EGFR Mut R1-6/L417H and comparing their anchorage-independent growth. We selected a NIH 3T3 sub-line that has an unusually low expression of endogenous EGFR (Wikstrand et al., 1995), and the EGFR constructs we used for stable expression contained no GFP tag.

Selected clones stably expressing wt EGFR, EGFR Mut R1-5, and EGFR Mut R1-6, were found to express their respective EGFR proteins to similar extents, while levels of ER-retained EGFR L417H and EGFR Mut R1-6/L417H are somewhat lower (Figure 2.4*A*). However, in all five cases, robust over-expression of EGFR was observed to be far above the endogenous level of EGFR expressed by the parent NIH 3T3 cell line (vector control; Figure 2.4*A*), and cellular distributions are similar to those proteins transiently expressed in RBL-2H3 cells (compare Figs. 2.1*B* and 2.4*B*). Endo H sensitivity further shows that these stably expressed EGFR proteins localize in NIH 3T3 cells similarly to their transiently expressed counterparts in RBL-2H3 cells: wt EGFR and EGFR Mut R1-6 are largely trafficked out of the ER, whereas EGFR mutants that contain L417H are localized exclusively in the ER (compare Figure 2.4*C* to Figure 2.2*B*).

We tested the stably expressed receptors for their capacity to induce anchorage independent growth, a reliable indicator of transformation (Macpherson and Montagnier, 1964). Although the NIH 3T3 cells stably expressing vector alone exhibit small amounts of endogenous EGFR, these levels are not sufficient to mediate anchorage-independent growth, even when stimulated with EGF. Overexpression of wt EGFR results in robust transformation, but only in the presence of EGF (Figure 2.5 *A* and *B*). Similarly, over-expressed EGFR Mut R1-5 transforms NIH 3T3 cells in an EGF-dependent manner. However, stably expressed EGFR Mut R1-6 stimulates the anchorage independent growth of NIH 3T3 cells even in the absence of EGF. Furthermore, EGFR Mut R1-6/L417H retains the capacity to transform cells in a ligand-

Figure 2.4. *EGFR Mut R1-6/L417H is retained in the ER when stably expressed in NIH 3T3 cells.* (**A**) Whole cell lysates of NIH 3T3 cells stably expressing wt EGFR, EGFR Mut R1-5, EGFR Mut R1-6, EGFR L417H, EGFR Mut R1-6/L417H, or vector only were subjected to Western blot analysis using an anti- EGFR antibody and an anti-actin antibody (loading control). (**B**) Confocal images of NIH 3T3 cells stably expressing vector only, wt EGFR, EGFR Mut R1-6, EGFR L417H, or EGFR Mut R1-6/L417H. Prior to imaging, cells were fixed and labeled with an anti-N-terminal EGFR antibody followed by Alexa 647 anti-mouse IgG, then washed, permeabilized, and labeled with anti-C-terminal EGFR antibody followed by Alexa 488-anti-rabbit IgG. (**C**) Whole cell lysates from NIH 3T3 cells stably expressing either the wt EGFR, EGFR Mut R1-6, EGFR Mut R1-6/L417H, or EGFR L417H were untreated, or were treated with Endo H or with PNGase F for 24 hours before Western blot analysis with an anti-EGFR antibody.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R1-5
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EH
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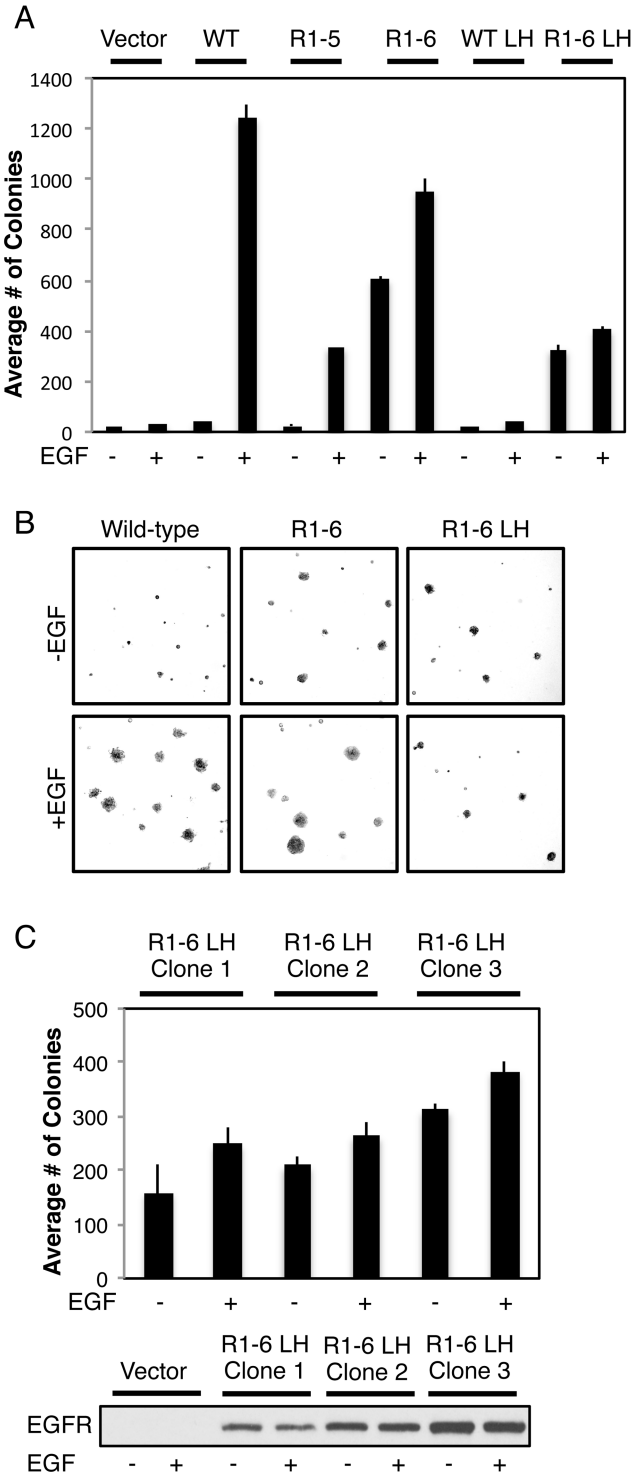
EGFR

Figure 2.5. *ER-retained EGFR MutR1-6/L417H transforms cells in a growth factor-independent manner.* (**A**) NIH 3T3 fibroblasts stably expressing vector, wt EGFR, EGFR Mut R1-5, EGFR Mut R1-6, EGFR L417H, or EGFR Mut R1-6/L417H, incubated with or without EGF (100 ng/ml), were cultured in soft agar. The data shown represent the mean ± SD for colonies per 2.25 cm^2 from at least three independent experiments. ***, $P<0.001$. (**B**) Representative images of the colonies quantified in **A**. (**C**) Three different clones of NIH 3T3 fibroblasts stably expressing EGFR Mut R1-6/L417H cultured in soft agar as in **A** (Mean ± SD from three independent experiments). Western blot analysis using an anti-EGFR antibody was performed on whole cell lysates from the same three clones and vector control cells, without or with EGF stimulation to determine relative expression levels.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R1-5
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EGF - + - + - + - + - + - +
B
Wild-type
R1-6
R1-6 LH
-EGF
+EGF
C
R1-6 LH Clone 1
R1-6 LH Clone 2
R1-6 LH Clone 3
Average # of Colonies
500
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100
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Vector
R1-6 LH Clone 1
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R1-6 LH Clone 3
EGFR
EGF - + - + - + - + - + - +

independent manner, whereas EGFR L417H is incapable of transforming NIH 3T3 fibroblasts in the absence or presence of ligand. The transforming capacity of EGFR Mut R1-6/L417H is observed in multiple clones, and the degree of colony formation by these clones correlates with EGFR Mut R1-6/L417H expression levels (Figure 2.5*C*). Likewise, EGFR Mut R1-6, which expresses to a higher level than EGFR Mut R1-6/L417H (Figure 2.4*A*), forms more colonies than this ER-retained mutant in soft agar in the presence and absence of EGF (Figs. 2.5*A* and *B*). Collectively, these results show that the charge silencing mutation R1-6 in the JX region leads to structural alterations in EGFR that cause ligand-independent transformation by receptors at the PM and the ER.

We observe that EGF consistently enhances the number of colonies formed in NIH 3T3 cells stably expressing EGFR Mut R1-6/L417H by a small, but statistically significant, amount (Figure 2.5*A*). Thus, we tested the possibility that a small population of EGFR Mut R1-6/L417H, below the level of detection by imaging (Figure 2.4*B*) and Endo H sensitivity assays (Figure 2.4*C*), is trafficked to the PM. Anchorage independent growth assays were carried out on the cells expressing EGFR Mut R1-6/L417H in the presence of the anti-EGFR antibody Cetuximab (mAb 225), which prevents the transition of EGFR to its active conformation (Li et al., 2005). Ligand-independent transformation mediated by EGFR Mut R1-6/L417H that is due to a PM-associated pool would be expected to be reduced in the presence of this antibody. However, addition of Cetuximab did not decrease transformation mediated by EGFR Mut R1-6/L417H under conditions in which it significantly inhibited transformation by wt EGFR (Figure 2.6). These findings suggest that the enhanced transformation seen in NIH 3T3 cells stably expressing the EGFR Mut R1-6/L417H in the presence of EGF is due to signaling by endogenous EGFR at

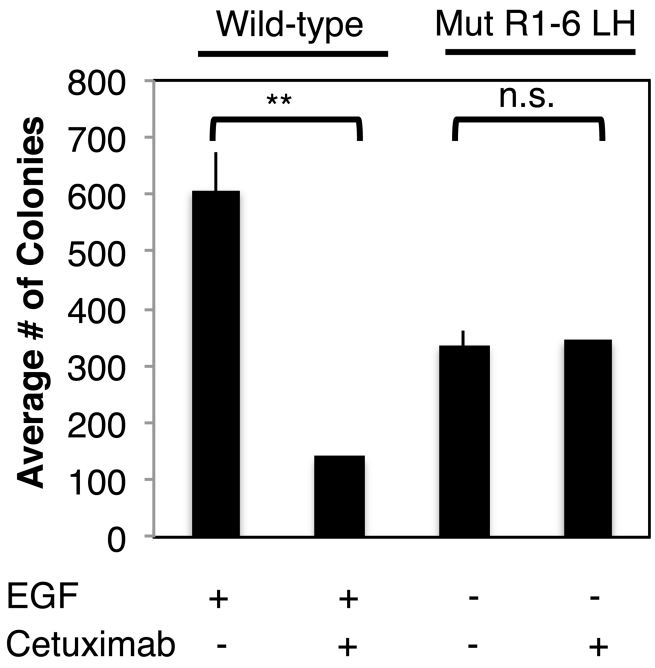


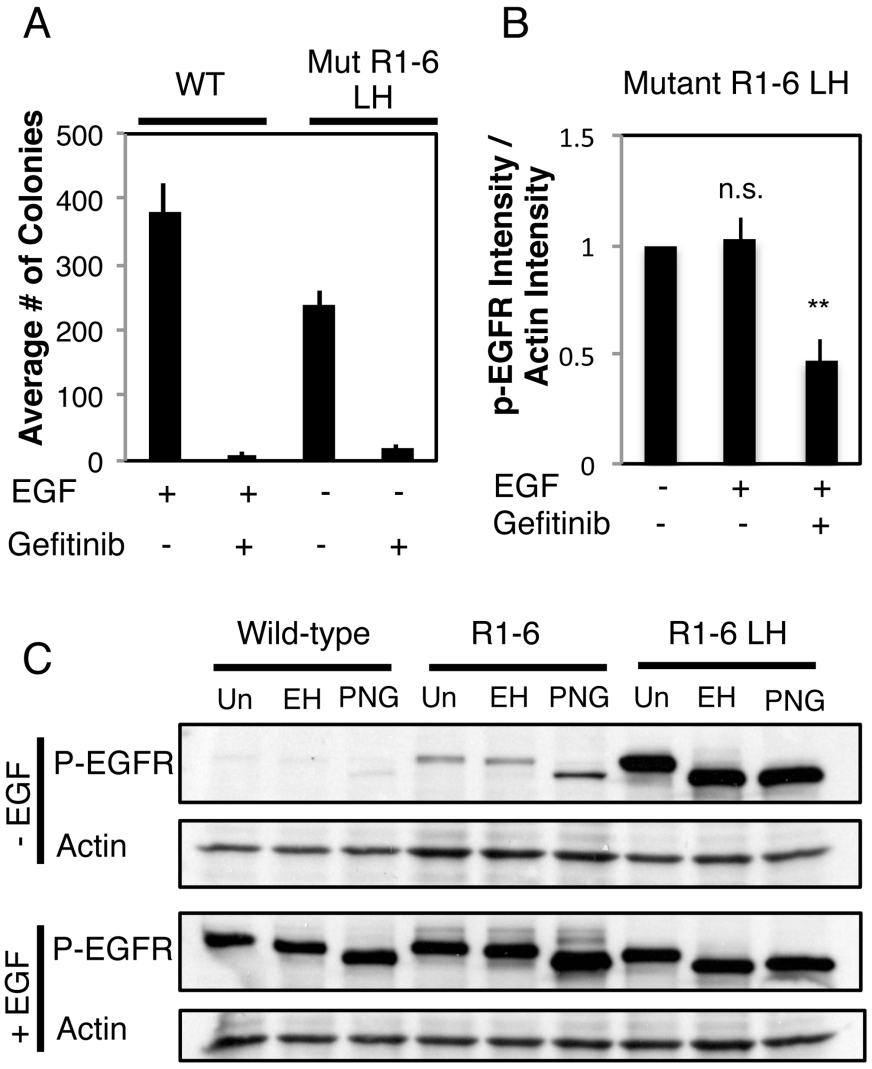
Figure 2.6. *Anti-EGFR Cetuximab does not inhibit transformation by EGFR Mut R1-6/L417H.* NIH 3T3 cells stably expressing wt EGFR or EGFR Mut R1-6/L417H were cultured in soft agar in the presence or absence of Cetuximab (5 μg/ml) and / or EGF (20 ng/ml). Mean ± SD from three independent experiments, **, $P<0.01$; n.s., not significant.

the PM that synergizes with activity of constitutively active EGFR Mut R1-6/L417H at the ER (see Discussion).

2.4.3 Transformation mediated by ER-retained EGFR Mut R1-6/L417H requires its kinase activity.

We investigated whether EGFR Mut R1-6/L417H signals to cytoplasmic proteins to transform cells. We found that treatment with the EGFR tyrosine kinase inhibitor, gefitinib (Iressa) at 2.5 μM eliminates transformation mediated both by NIH 3T3 cells expressing EGFR Mut R1-6/L417H and by EGF-stimulated NIH 3T3 cells overexpressing wt EGFR (Figure 2.7*A*). The effects of gefitinib on both of these stable cell lines is dose-dependent, with IC_{50} values of < 2 μM gefitinib (data not shown). We also observed significant basal phosphorylation of EGFR Mut R1-6/L417H by Western blotting of whole cell lysates from cells expressing this mutant receptor using anti-phospho-EGFR (Y1068) antibody. This phosphorylation is not detectably enhanced upon EGF addition, and decreases substantially upon treatment with gefitinib (Figure 2.7*B*). These results suggest that the ligand-independent transformation mediated by the ER-retained EGFR Mut R1-6/L417H is consistent with its constitutive activity. Wt EGFR (and EGFR L417H) exhibits a lower level of constitutive phosphorylation at residue 1068, and Endo H digestion analysis confirms that the wt EGFR that becomes robustly phosphorylated at Y1068 upon the addition of ligand is not in the ER, but rather is located at the PM (Figure 2.7*C*). In contrast, Y1068-phosphorylated EGFR Mut R1-6/L417H is retained in the ER (Figure 2.7*C*), providing additional evidence that receptor signaling from this location leads to transformation.

Fig. 2.7. *Transformation by ER-retained EGFR Mut R1-6/L417H and its ligand-independent phosphorylation are EGFR kinase-dependent.* (**A**) Inhibition of colony formation of NIH 3T3 clones by the EGFR kinase inhibitor gefitinib (2.5 μM). Data shown are the mean ± SD from three independent experiments. ***, P<0.001; ****, P<0.0001. (**B**) Quantification of phospho-Y1068 EGFR detected in the whole cell lysates from serum starved NIH 3T3 cells expressing EGFR Mut R1-6/L417H, treated as shown (gefitinib = 5 μM). Data were averaged from four independent experiments. Error bars show S.E., **, P<0.01, n.s., not significant. (**C**) Whole cell lysates from serum-starved NIH 3T3 cells stably expressing either wt EGFR, EGFR Mut R1-6, or EGFR Mut R1-6/L417H were treated without or with EGF as in Fig. 3. Lysates (equal protein content) were untreated or were treated with Endo H, or with PNGase F for 24 hours before Western blot analysis using anti-phospho-Y1068-EGFR and anti-actin antibodies.


A
WT
Mut R1-6 LH
Average # of Colonies
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EGF + + - -
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Mutant R1-6 LH
p-EGFR Intensity / Actin Intensity
1.5
1
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EGF - + +
Gefitinib - - +
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Wild-type
R1-6
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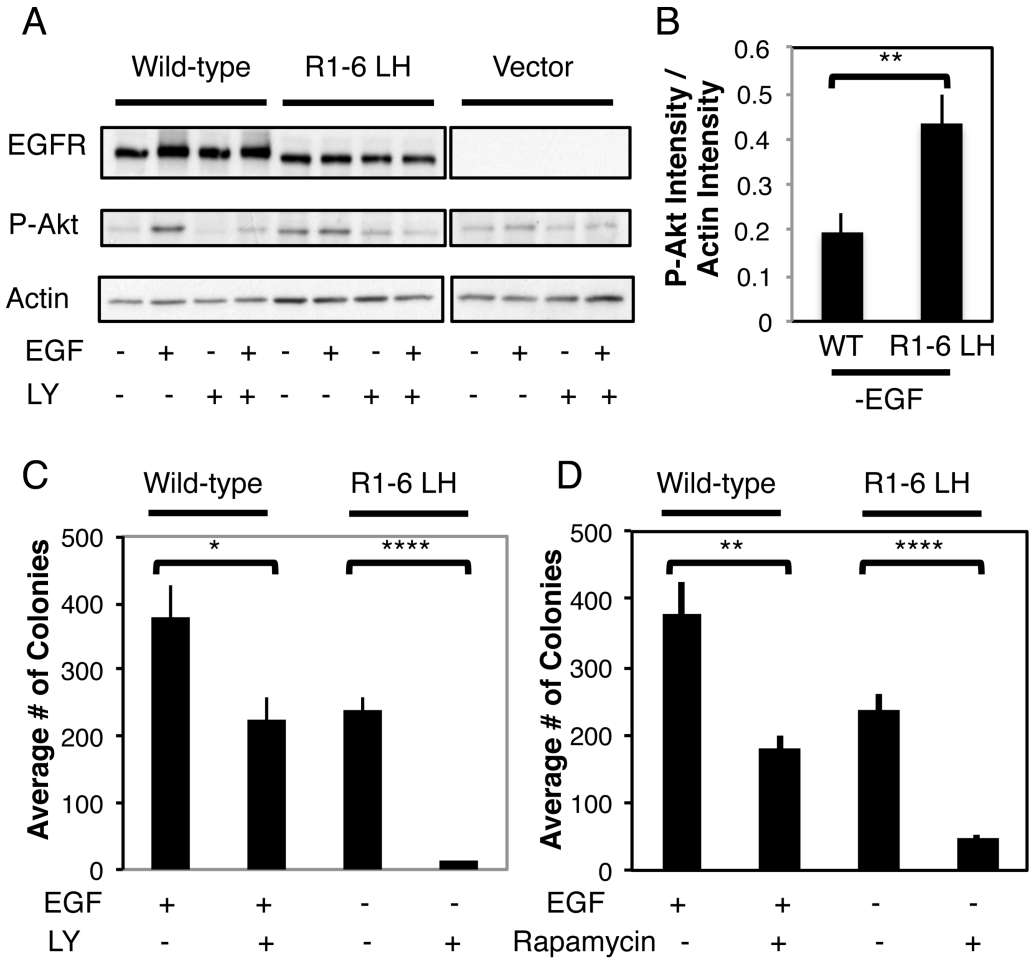
- EGF
P-EGFR
Actin
+ EGF
P-EGFR
Actin

2.4.4 The ER-retained EGFR Mut R1-6/L417H signals through the PI3K pathway.

To determine the signaling pathway(s) responsible for mediating the ligand-independent transforming activity of EGFR Mut R1-6/L417H, we focused our investigation on proteins that interact with EGFR via phospho-Y1068, including PI3K. Figure 2.8 summarizes results comparing the activity of this pathway in the NIH3 3T3 cells stably expressing wt EGFR to those expressing EGFR Mut R1-6/L417H. Cells expressing EGFR Mut R1-6/L417H exhibit higher basal levels (without EGF stimulation) of activated Akt than cells expressing wt EGFR (Figure 2.8 *A* and *B*), and this activation is prevented by treatment with the PI3K inhibitor LY294002 at 10 μM (Figure 2.8*A*). Furthermore, treatment with a concentration of LY294002 (5 μM) that only partially decreases EGF-dependent wt EGFR-mediated anchorage-independent cell growth abolishes colony formation mediated by EGFR Mut R1-6/L417H, suggesting greater sensitivity of transformation by this mutant to PI3-kinase inhibition (Figure 2.8*C*). Similarly, transformation mediated by EGFR Mut R1-6/L417H is more susceptible to rapamycin, an inhibitor that interferes with the capacity of PI3K to signal through mTOR, than transformation mediated by wt EGFR (Figure 2.8*D*). In contrast, cells expressing EGFR Mut R1-6/L417H do not show elevated levels of phosphorylated Erk compared to wt EGFR-expressing and vector control cells, with or without EGF stimulation, and treatment with the MEK inhibitor PD98059 inhibits transformation mediated by wt EGFR and EGFR Mut R1-6/L417H to a similar extent (Figure 2.9). These results provide a consistent picture that the transformation caused by ER-retained EGFR Mut R1-6/L417H is mediated primarily by the PI3K-mTOR signaling axis.

Figure 2.8. *Transformation mediated by ER-retained EGFR Mut R1-6/L417H depends on PI3K signaling.* (**A**) Whole cell lysates from serum-starved NIH 3T3 cells stably expressing wt EGFR or EGFR Mut R1-6/L417H and treated as indicated were immunoblotted with antibodies specific for phospho-Akt S473, total EGFR, or actin (loading control). Before lysing the cells, EGF was added for 5 min at 100 ng/ml in the presence or absence of 10 μM LY294002 (LY). (**B**) Quantification of Akt phosphorylation in cells expressing wt EGFR or EGFR Mut R1-6/L417H in the absence of EGF. Data averaged from five independent experiments including that shown in **A** (error bars show S.E., **, $P<0.01$). (**C**) The effect of the PI3K inhibitor LY294002 (5μM) on the capacity of wt EGFR or EGFR Mut R1-6/L417H-expressing cells to grow in soft agar. The data shown represent the mean ± SD from three independent experiments. *, $P<0.05$; ****, $P<0.0001$. (**D**) The effect of mTOR inhibition on the capacity of wt EGFR or EGFR Mut R1-6/L417H-expressing cells to grow in soft agar was assessed by adding rapamycin (25 nM) to soft agar cultures. Mean ± SD from three independent experiments, **, $P<0.01$; ****, $P<0.0001$.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0.5
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Wild-type
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Average # of Colonies
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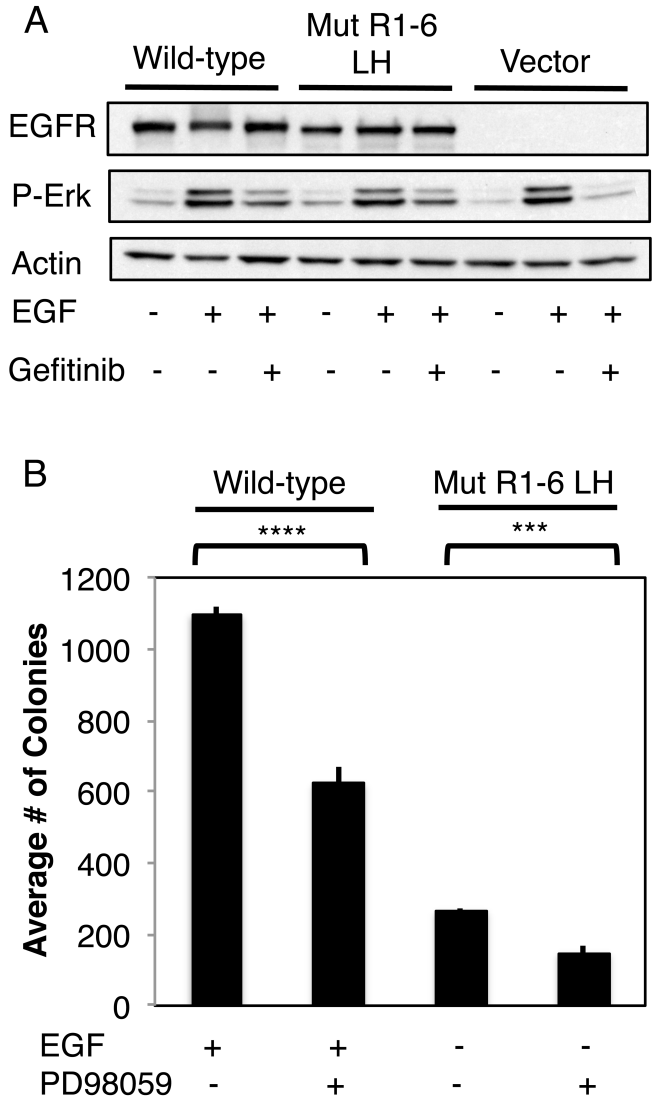


Figure 2.9. *Contribution of Erk pathway to EGFR Mut R1-6/L417H-mediated transformation.* (**A**) Whole cell lysates of serum-starved NIH 3T3 cells stably expressing vector control, wt EGFR, or EGFR Mut R1-6/L417H were treated or not with EGF (100 ng/ml) and or gefitinib (5μM) and immunoblotted with antibodies against phospho-Erk, total EGFR, and actin (loading control). (**B**) Inhibition of transformation by the MEK inhibitor PD98059 (50 μM). Mean ± SD from three independent experiments, ***, P<0.001; ****, P<0.0001.

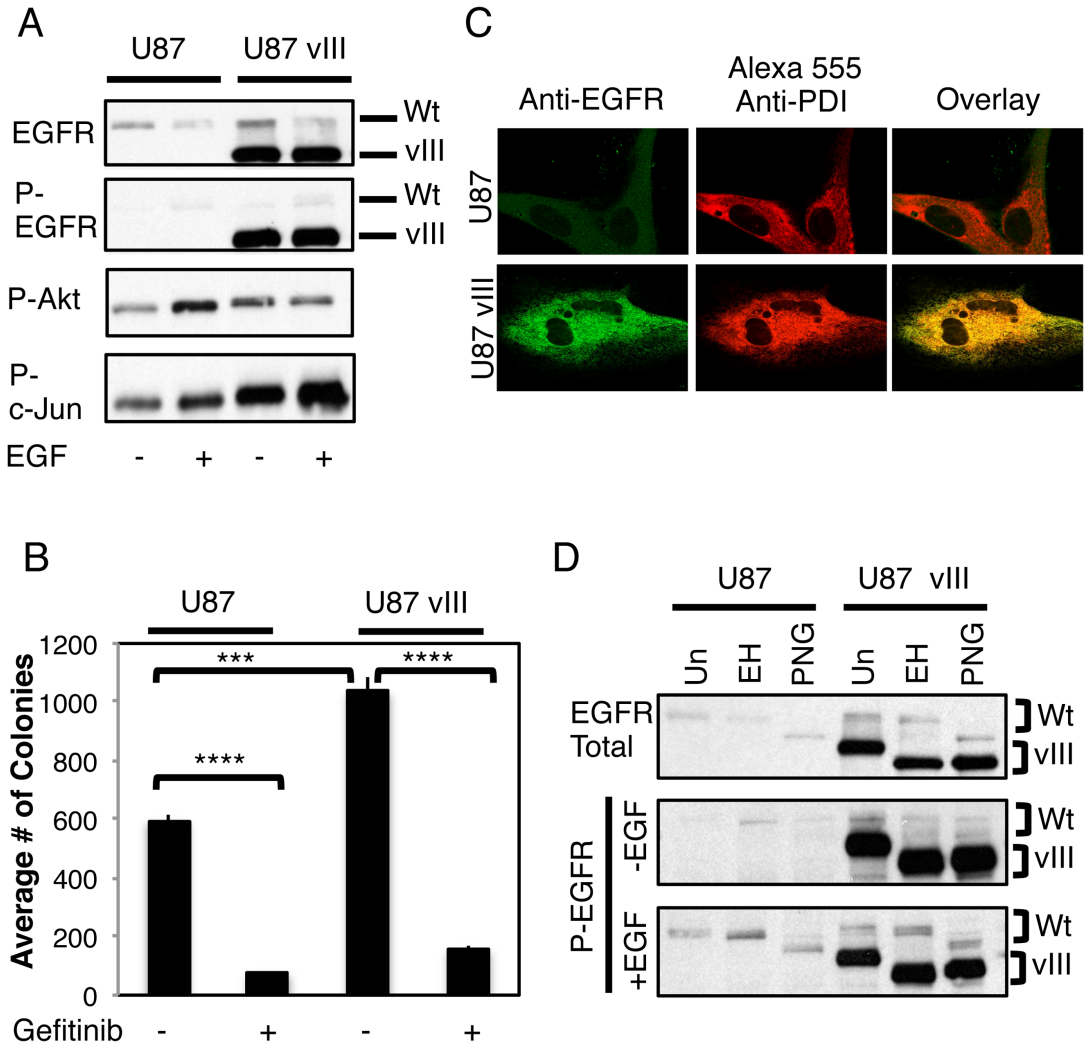
2.4.5 EGFRvIII localizes to the ER where it is constitutively active in U87 cells.

Because the subcellular distribution of EGFRvIII in cancer cells has been controversial, we investigated this question by generating U87 human glioblastoma cells that stably express EGFRvIII. Similar to EGFR Mut R1-6/L417H in NIH 3T3 cells, we found that EGFRvIII in U87 cells is robustly phosphorylated on tyrosine 1068 (Y1068), independent of EGF, and that Akt and c-Jun, components of the PI3K signaling pathway, are also basally phosphorylated (Figure 2.10*A*). U87 cells expressing EGFRvIII show enhanced transformation compared to vector control, as revealed by anchorage independent growth assays in soft agar, and this colony formation is inhibited by treatment with gefitinib (Figure 2.10*B*). Immunochemical labeling of EGFRvIII with an antibody specific for the cytoplasmic C-terminus of EGFR showed a clear reticulate pattern that colocalizes with PDI (Figure 2.10*C*). ER-localization of EGFRvIII in U87 cells was further confirmed by complete sensitivity of this mutant receptor to digestion by Endo H (Figure 2.10*D*). Furthermore, Y1068-phosphorylated EGFRvIII is Endo H sensitive, corresponding to ER localization, whereas Y1068-phosphorylated wt EGFR is not, consistent with PM localization of this receptor (Figure 2.10*D*). These results demonstrate that EGFRvIII, like EGFR R1-6/L417H, can signal from the ER in a ligand-independent manner.

2.5 Discussion

The concept of signal generation from phosphorylated RTKs that are activated by their respective ligands at the PM has been a tenet in the field of receptor biology since the early 1980's (Ushiro and Cohen, 1980). The findings that many epithelial tumors overexpress RTKs, particularly members of the ErbB family, and that they are key contributors in cancer

Figure 2.10. *EGFRvIII stably expressed in U87 glioblastoma cells is robustly phosphorylated, enhances cell transformation, and is localized to the ER.* (**A**) Parental U87 cells and those stably expressing EGFRvIII were treated without or with 100 ng/ml EGF for 5 min. Whole cell lysates were electrophoresed and Western blots were probed with antibodies that recognize EGFR, or the activated forms of EGFR (phospho-Y1068-EGFR), Akt (P-T308) and c-Jun. (**B**) Parental U87 cells and those stably expressing EGFRvIII were cultured in soft agar without EGF, with or without gefitinib (20 μM). The data shown represent the mean ± SD for colonies per 2.25 cm^2 from at least three independent experiments. ***, P<0.001; ****, P<0.0001. (**C**) Parental U87 cells and those stably expressing EGFRvIII were fixed, permeabilized, and labeled with an anti-C-terminal EGFR antibody followed by Alexa 488-anti-rabbit IgG. Cells were then labeled with an anti-PDI antibody followed by Alexa555-anti-mouse IgG to label the ER. (**D**) Whole cell lysates from parental U87 cells and those stably expressing EGFRvIII treated without or with EGF (100 ng/ml) were untreated (Un) or treated with Endo H (EH) or with PNGase F (PNG) for 24 hours before Western blot analysis using an anti-EGFR antibody or an anti-phospho-Y1068-EGFR antibody.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Alexa 555
Anti-PDI
Overlay
U87
U87 vIII
B
U87
U87 vIII
Average # of Colonies
1200
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200
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EGFR
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P-EGFR
-EGF
Wt
vIII
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vIII

progression (Clark et al., 1984), led to an explosion of a field that continues to grow at a rapid pace today. By the end of that decade it was understood that, like overexpression, mutations in RTKs can lead to dysregulated signaling. A prominent example in human brain cancers is EGFRvIII, a deletion mutant lacking exons 2-7 (Humphrey et al., 1988). While a number of other cancer-causing mutations within surface-localized RTKs have been described previously (Lemmon and Schlessinger, 2010), impaired receptor trafficking both to and from the PM has been recognized more recently as yet another possible mechanism of dysregulation (Choudhary et al., 2009; Carpenter and Liao, 2009).

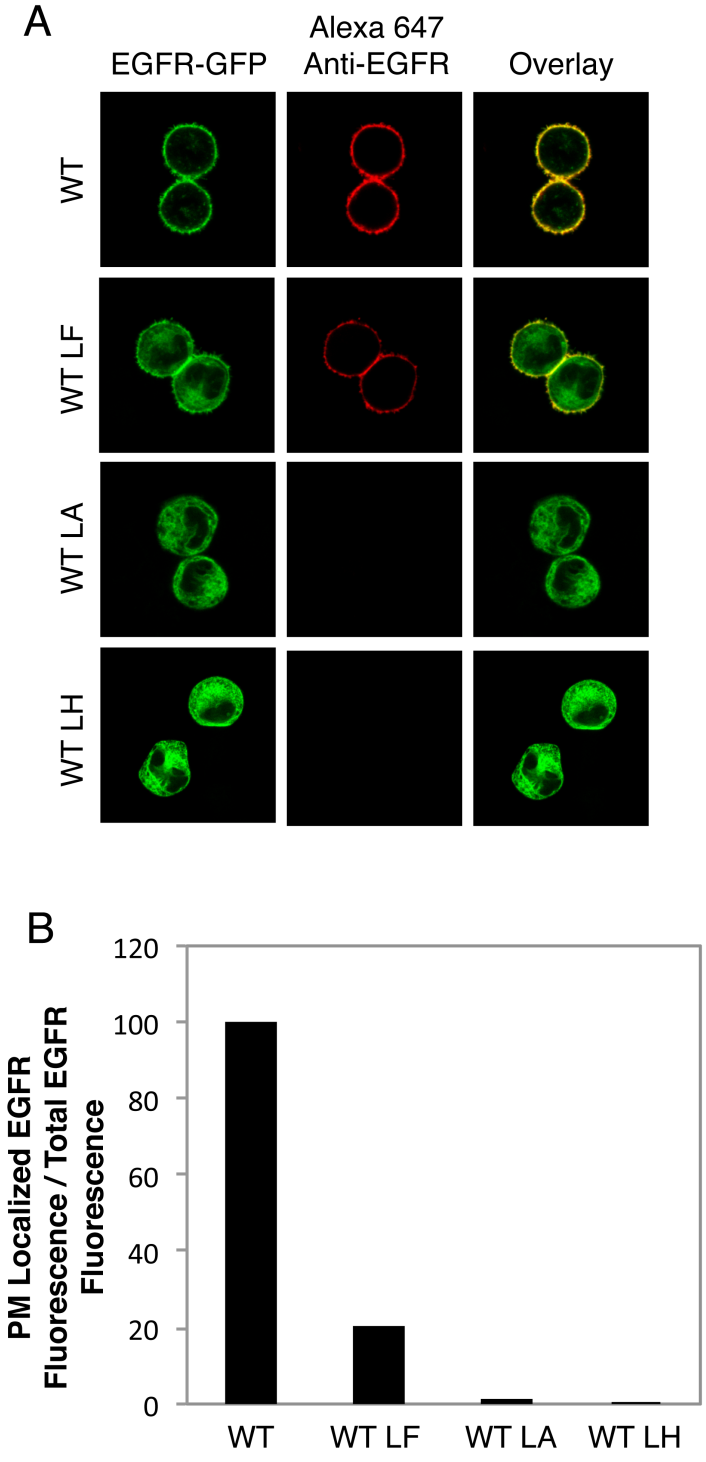
Prompted by the hypothesis that the basic residues in the JX region of EGFR negatively regulate kinase domain interactions, we sequentially mutated these residues to alanines. We found that, by reducing the net charge of the polybasic region within the JX region of EGFR from +8 to +3, we generated a receptor (EGFR Mut R1-6) that is constitutively active and can transform cells even when retained in the ER by a point mutation (L417H; Figs. 2.5 and 2.7). We showed that EGFR Mut R1-6/L417H receptors are intracellularly retained using both confocal microscopy and quantitative flow cytometry (Figs. 2.1 and 2.4), and we identified the site of retention as the ER both by colocalization with the ER-marker PDI (Figure 2.2*A*) and by Endo H digestion (Figs. 2.2*B* and 2.4*C*). Similarly, we demonstrate that EGFRvIII in U87 cells is completely Endo H sensitive (Figure 2.10*D*) and that its immunolabeling colocalizes with PDI (Figure 2.10*C*), consistent with its ER localization. Furthermore, we show that both EGFRvIII (Figure 2.10) and EGFR Mut R1-6/L417H (see below) are constitutively phosphorylated in the ER and signal primarily through the PI3K pathway to mediate cell transformation.

Although ER-retained RTK mutants have been described previously (Ekstrand et al., 1995; Privalsky et al., 1984), these mutants all possess a large deletion of the original protein

sequence. Novel to this study is the discovery that a single amino acid substitution of leucine 417 for histidine results in the retention of EGFR in the ER. The causative factor for retention seems to be the loss of leucine 417, as replacement of this residue with alanine also results in complete ER-retention, and replacement with a phenylalanine results in dramatically reduced PM association (Figure 2.11). Leucine 417 is located in domain III of the extracellular region of EGFR, which resembles other leucine-rich repeat proteins (Bajaj et al., 1987; Ward and Garrett, 2001). We hypothesize that replacement of leucine 417 with a histidine or alanine residue disrupts the closely packed hydrophobic core of the leucine-rich repeat, and this disruption results in a structural change, which in turn disallows the exit from the ER of EGFRs containing this mutation. Interestingly, human ErbB3 and ErbB4 both have a hydrophobic phenylalanine at position 417 (Ward and Garrett, 2001), consistent with the observed tolerance for this substitution in EGFR.

It has been suggested that electrostatic binding of the JX region and a TKD face to acidic lipids in the PM may restrict access of the kinase domain to substrate tyrosines in inactive conformations (McLaughlin et al., 2005). Recently reported molecular dynamics simulations suggest that basic residues in the JX region of EGFR associate electrostatically with acidic lipids and can contribute to burying hydrophobic residues of the JX region of inactive EGFRs in the membrane (Arkhipov et al., 2013). We hypothesized that an EGFR mutant lacking these basic amino acids has a more easily accessible kinase domain that signals without regulation. Consistent with this view, we showed that stable expression of EGFR Mut R1-6 confers the capacity of NIH 3T3 cells to form colonies under anchorage-independent and ligand-independent conditions, irrespective of its localization at the PM or ER (Figure 2.5). Notably, stable expression of EGFR Mut R1-5 does not result in ligand-independent transformation, indicating

Figure 2.11. *EGFR-GFP L417A is ER-retained, whereas EGFR-GFP L417F shows reduced PM association.* (**A**) Expression of wt and mutant EGFR-GFP constructs in suspended RBL mast cells. Transiently expressing cells were fixed and labeled with an anti-N-terminal EGFR antibody, followed by Alexa647-anti-IgG to detect PM-associated EGFR. (**B**) Quantification of the effect of the L417H (LH), L417F (LF), and L417A (LA) point mutations on biosynthetic trafficking. RBL-2H3 cells transiently expressing GFP-tagged EGFR constructs were harvested, fixed and labeled with anti-N-terminal EGFR antibody followed by Alexa647-anti-IgG, then analyzed by flow cytometry to determine the ratio of PM-localized EGFR fluorescence to total-EGFR fluorescence. Data from mutants is normalized to wt and $\geq$8,000 cells expressing each construct were analyzed.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WT
WT LF
WT LA
WT LH

that reduction of the net charge of the polybasic JX region from +8 to +3 is the minimal change necessary for constitutive kinase activity and oncogenic potential of EGFR Mut R1-6. Furthermore, this constitutively active mutant maintains its activity and transforming ability when retained in the ER (Mut R1-6/L417H; Figure 2.5). We show that this ER-retained EGFR Mut R1-6/L417H is phosphorylated at this intracellular location (Figure 2.7*C*), and the extracellular antagonist Cetuximab does not inhibit this ligand-independent transformation (Figure 2.6). Interestingly, the number of colonies formed by EGFR Mut R1-6/L417H-expressing cells increases to a small but significant extent in the presence of EGF (Figure 2.5*A*). This may be explained by the small amount of EGFR that is endogenous to the parental NIH 3T3 cells, which is not sufficient to promote colony formation in soft agar in response to EGF, but may synergize with the large number of constitutively active Mut R1-6/L417H EGFRs. This synergy may involve additional activation of Erk by EGF (Figure 2.9).

Consistent with a constitutively activated state, EGFR Mut R1-6/L417H is phosphorylated at residue Y1068 independent of the addition of EGF (Figure 2.7 *B* and *C*). We are currently investigating the nature of this constitutive phosphorylated state, including the possibility that EGFR Mut R1-6/L417H forms dimers in the ER. Interestingly, two of the mutated residues, arginine 651 and lysine 652, are involved in the formation of an antiparallel coiled-coil that is predicted to be important for the formation of active dimers (Jura et al., 2009). Activation of EGFRvIII has been suggested to be independent of dimer formation (Chu et al., 1997); thus, understanding the basis for activation of EGFR Mut R1-6/L417H could reveal a potentially novel mechanism for EGFR dimerization that could be more generally relevant for cell transformation by EGFR variants.

Constitutively phosphorylated at Y1068 and oriented correctly across the ER-membrane (Figure 2.3), EGFR Mut R1-6/L417H appears poised to requisition the canonical cytosolic signaling proteins that normally interact with EGF-stimulated EGFR. Adaptors that interact with pY1068, Grb2 and Gab2 (Rojas et al., 1996; Kong et al., 2000), lead to Ras-Erk/MAPK and PI3K pathways, respectively. We found that EGFR Mut R1-6/L417H signals primarily through the PI3K pathway: Colony formation mediated by Mut R1-6/L417H EGFR is particularly sensitive to treatment with the PI3K inhibitor LY294002 (Figure 2.8*B*) and to mTOR inhibition by rapamycin (Figure 2.8*D*). Furthermore, we found that Akt is basally phosphorylated in EGFR Mut R1-6/L417H-expressing cells (Figure 2.8 *A* and *B*). In contrast, Erk phosphorylation is not increased in these cells compared to those expressing wt EGFR or vector only (Figure 2.9*A*). Although inhibition of Erk by PD98059 decreases colony formation mediated by EGFR Mut R1-6/L417H, this decrease is similar to that for wt EGFR in the presence of EGF (Figure 2.9*B*), suggesting a contribution from endogenous EGFR to the transformation phenotype. Interestingly, EGFRvIII has been shown to signal similarly through the PI3K pathway, while only slightly activating the Ras-Erk/MAP kinase pathway (Moscatello et al., 1996, 1998). Determining how ER-retained EGFR mutants selectively commandeer the PI3K pathway merits further study. We note that all Ras isoforms are tethered to membranes through an isoprenyl moiety together with either palmitoylation or a polybasic sequence (Hancock et al., 1990). In contrast, PI3K and its downstream mediator Akt are cytosolic proteins, which are recruited through protein-protein or protein-lipid interactions. This disposition likely increases their intracellular accessibility for activation by ER-membrane-localized EGFR Mut R1-6/L417H or EGFRvIII receptors. Our results confirm that wholly ER-retained, constitutively active EGFRs can drive cellular

transformation. In a clinical context, cancers mediated by similarly retained mutant EGFRs will likely be more optimally treated by inhibitors that access their intracellular location.

2.6 Acknowledgments

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Chapter 3

PtdIns4P synthesis by PI4KIIIα and its impact on proper biosynthetic protein trafficking

3.1 Abstract

The biosynthetic trafficking of receptors and other integral membrane proteins from the endoplasmic reticulum (ER) to the plasma membrane (PM) underlies the capacity of these proteins to participate in crucial cellular functions. Phosphoinositides have been shown to mediate distinct biological functions in cells, and phosphatidylinositol-4-phosphate (PI4P), in particular, has emerged as a key regulator of biosynthetic trafficking. To investigate the role of PI4P in orchestrating trafficking events, we developed a novel method to monitor the biosynthetic trafficking of transiently transfected proteins. We utilized pharmacological inhibition of different isoforms of PI 4-kinase to provide evidence for a distinct and necessary role for PI4P, synthesized by PI4KIIIα, in ER-to-PM trafficking. We complement our pharmacological studies with a genetic approach to deplete PI4P in the early biosynthetic pathway by overexpression of the Sac1 phosphatase. Taken together, these findings provide evidence that a specific pool of PI4P, synthesized by PI4KIIIα, plays a key role in biosynthetic trafficking of two different classes of proteins.

3.2 Introduction

Studies of the phosphorylated derivatives of phosphatidylinositol (PI) have shown that these molecules possess distinct biological functions and localize selectively to organelles (reviewed by Di Paolo and De Camilli, 2006). Due to variable phosphorylation of hydroxyl

groups on their inositol rings, seven different interconvertable phosphoinositide species exist in cells, including phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol 4,5 bisphosphate (PIP_2). Each phosphoinositide species is enriched in specific intracellular membranes; for example, $PI(4,5)P_2$ is highly localized to the inner leaflet of the PM, whereas PI4P is enriched at the Golgi complex.

$PI(4,5)P_2$ is a well-established regulator of multiple cellular processes including vesicle trafficking, phagocytosis (Coppolino et al., 2002), membrane ruffling (Honda et al., 1999), cell motility, cell adhesion (Ling et al., 2002), and regulation of ion channel activity and receptor phosphorylation (Hilgemann et al., 2001; Michailidis et al., 2011). In addition, $PI(4,5)P_2$ is the substrate for generation of the second messengers inositol tris-phosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1983), and is thus necessary for agonist-stimulated Ca^{2+} signaling. Furthermore, there have been several reports of $PI(4,5)P_2$ existing in functionally and spatially distinct pools in the PM that support specific signaling platforms (Lui et al., 1998; Johnson et al., 2008; Vasudevan et al., 2009; Calloway et al., 2011). PI4P, the most prevalent mono-phosphorylated PI-derivative in cells (Lemmon, 2008), was for many years believed to serve no function outside of being the precursor for PIP_2. Recently, however, a number of PI4P-dependent processes have been characterized, in particular its role in the regulation of protein trafficking (Figure 1.4). For example, PI4P is required to promote COPII-mediated ER export of proteins at ER exit sites (Blumental-Perry et al., 2006), and, by interacting with CERT, OSBP, and FAPP lipid transfer proteins (collectively termed COFs), PI4P also plays a role in sphingolipid and sterol metabolism (D'Angelo, 2008).

Organelle specific phosphoinositide distributions are maintained by the tight regulation of PI-kinases and PI-phosphatases. Four distinct PI 4-kinases have been described in mammalian

cells, including type II (PI4KIIα and PI4KIIβ) and type III (PI4KIIIα and PI4KIIIβ) kinases (Balla and Balla, 2006). The type II PI-4kinases are palmitoylated (Barylko et al., 2001) and thus strongly membrane associated, particularly in the trans-Golgi apparatus (Wang et al., 2003) and to a lesser extent in endosomes (Balla et al., 2004). PI4KIIIβ localizes primarily to the Golgi apparatus (Wong et al., 1997), as detected by its coincidence with Arf1, a small GTP-binding protein (Godi et al., 1999). While the molecular details of how these enzymes are linked to Golgi-derived biosynthetic transport remain unknown, they have all been implicated with proper Golgi function and secretion (Balla and Ball, 2006).

The predominant localization of PI4KIIIα is not well understood. Mammalian PI4KIIIα is the homolog of yeast Stt4, a PM-associated protein (Audhya and Emr, 2002) that has more recently been suggested to localize to specific regions called PI-kinase (PIK) patches, important for signaling (Baird et al., 2008). Paradoxically, although Stt4 is not commonly detected in the ER in yeast, the PI4P pool generated by this kinase is mainly dephosphorylated by the ER-localized phosphoinositide phosphatase, Sac1p (Foti et al., 2001). The localization of PI4KIIIα to the ER is further supported by the presence of a FFAT (two phenylalanines [FF] in an acidic track) motif that binds to the integral ER proteins Scs2/22 in yeast, or VAP proteins in mammals (Stefan et al., 2011). In mammalian cells, PI4KIIIα has been reported to localize primarily to the ER based on immunofluorescence (Wong et al., 1997). However, more recent functional studies have called this localization into question. For example, PI4KIIIα has been reported to be responsible for the generation of hormone-sensitive phosphoinositide pools in the PM (Balla et al., 2008) and the PI4P generated by the largely cytoplasmically localized PI4KIIIα has been proposed to play a major role in defining PM identity (Nakatsu et al., 2012).

Our results support the view that a pool of PI4P synthesized by PI4KIIIα is functionally required for proper ER-to Golgi trafficking. Using a novel technique that we designed to monitor biosynthetic trafficking, we demonstrate that pharmacological inhibition of PI4KIIIα results in ER-retention of both the epidermal growth factor receptor (EGFR) and a GPI-anchored protein. Furthermore, we demonstrate that specific inhibition of a Golgi-localized PI 4-kinase does not result in ER-retention. Finally, we show that depletion of PI4P via overexpression of the PI-phosphatase Sac1 similarly inhibits protein trafficking to the PM. Taken together, these findings provide evidence that a specific pool of PI4P, synthesized by PI4KIIIα, is essential for proper biosynthetic protein trafficking.

3.3 Materials and Methods

3.3.1 Materials and Expression Plasmids

All cell culture reagents and all Alexa-dye conjugated secondary antibodies were from Invitrogen. FuGene HD was from Promega. The anti-N-terminal human EGFR antibody ((clone LA1) used for flow cytometry and immunochemistry) was from Millipore Corp. Anti-PDI (protein disulfide isomerase) mAb was from Affinity Bioreagents and the anti-GM130 was from BD Biosciences. PIK-93 was purchased from Symansis; wortmannin and brefeldin-A were from Calbiochem. Anti-GFP mAb, phenylarsine oxide (PAO), quercetin as well as any chemical not noted otherwise were purchased from Sigma-Aldrich Chemical Co. The human EGFR-GFP construct has been described previously (Monick et al., 2005). The Sac1-GFP construct was kindly provided by Y. Mao (Cornell University). YFP-GPI is a GPI-anchored protein containing yellow fluorescent protein and a consensus *N*-glycosylation site fused to the GPI-attachment signal of lymphocyte-function-associated antigen 3 (LFA-3) (Keller et al, 2001).

3.3.2 Cell Culture

RBL-2H3 mast cells were grown in MEM containing 20% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals) and 10 μg/ml gentamicin sulfate as described previously (Gosse et al., 2005). In preparation for imaging or flow cytometry, cells were plated at 25-50% confluency in 35 mm MatTek wells (MatTek Corporation) or 60 mm dishes, respectively. After approximately 20 h, RBL-2H3 cells were transfected with either mutant or wild-type versions of EGFR. These constructs were transfected using Fugene HD (Promega) per manufacturers' instructions, with modification to enhance transfection efficiency in the RBL cells previously described (Gosse et al., 2005). Cells were processed for imaging or flow cytometry 24 h after transfection.

3.3.3 Biosynthetic Trafficking Method

On day one, approximately $1x10^6$ RBL-2H3 cells were plated in 60mm dishes or $3.5x10^5$ RBL-2H3 cells were plated in 35-mm coverslip dishes (MatTek Corporation, Ashland, MA). On day two, cells were transfected using Fugene HD as described above. Cells were allowed to recover from transfection for 30 min to 1 hr at 37°C in normal RBL media before it was buffered with 40 mM HEPES, and the dishes were then sealed with parafilm and incubated at room temperature in the dark for 12 to 14 hr. On day three, the media was changed to normal RBL-2H3 media and cells were placed at 37°C; this point is time zero in the time course. For most experiments (as denoted in legends), perturbants, diluted in BSS (BSS: 20 mM HEPES, 135 mM NaCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5.6 mM glucose, 1 mg/ml BSA, pH 7.4), were added at hour 3 of the time course (media was replaced with BSS for control samples at this time in parallel). Samples from each time point were processed for confocal fluorescence microscopy or flow cytometry, as described below.

3.3.4 Confocal Fluorescence Microscopy

RBL-2H3 cells were washed once with BSS, fixed using 4% paraformaldehyde with 0.1% gluteraldehyde, permeabilized (or not) with 1% v/v Triton X-100, and labeled for 1 hour with specified antibodies in phosphate buffered saline (PBS) with 10 mg/ml BSA and 0.01% w/v sodium azide (PBS/BSA). Images were collected using an upright Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Exton, PA) with a 63 x 0.9 NA, HCX APO L U-V-I water-immersion objective.

3.3.5 Flow Cytometry

RBL-2H3 cells were harvested, washed once with BSS, fixed using 4% paraformaldehyde with 0.1% glutaraldehyde, and washed once with PBS/BSA. Cells expressing GFP-tagged wild-type EGFR or YFP-GPI constructs were labeled with appropriate antibodies for 1 hr in PBS/BSA. Samples were evaluated using a Becton Dickinson LSR II flow cytometer, and data were analyzed using BD FACSDiva software. Analysis was gated to include single cells on the basis of forward and side light-scatter, and data from single-color samples were used to determine the gates for positive fluorescence from each fluorophore.

3.3.6 Degranulation: β-Hexosaminidase Release

Cells were sensitized with IgE and plated in triplicate at a density of $5x10^5$/well and incubated overnight. The next day, cells were treated with PIK-93 (250nM or 1μM) for 40min in BBS without BSA, and β-hexosaminidase release was assessed as described previously (Naal et al., 2004).

3.3.7 Data analysis

Statistical analyses were determined using GraphPad Prism (GraphPad Software, La Jolla, CA), using the Student *t* test, with $p \leq 0.05$ considered statistically significant.

3.4 Results

3.4.1 Flow cytometry of transiently transfected cells to assess perturbations of early ER-to-Golgi trafficking events.

To manipulate the early stages of biosynthetic trafficking, we developed a protocol in which cells are incubated at 24°C for 14 hr following transient transfection with EGFR-GFP. This incubation at low temperature allows for protein synthesis to occur without subsequent trafficking to the PM. The next day, the cells are transferred to 37°C, at which time biosynthetic trafficking begins and the increased presence of EGFR at the PM can be quantified over time (Figure 3.1*A*). Representative confocal images of the flow cytometry samples (Figure 3.1*A*) show that following transfer to 37°C, it takes ~six hours until EGFR begins to accumulate visibly at the PM (Figure 3.1*B*). In agreement with previous reports on the timescale of EGFR protein maturation (Verheijden et al., 1992), at 2 hr most cells show predominantly ER-localized EGFR, and by 4 hr significant Golgi localization is observed, with some receptor localization at the PM (Figure 3.1*B*).

Preliminary experiments indicated that in order to observe the effects of potential inhibitors of ER-to-Golgi trafficking, a method in which a substantial percentage of transfected protein makes this transition at once is necessary. To determine if our flow cytometry method was suitable, we first tested the effects of Brefeldin A (BFA), a well-documented inhibitor of early biosynthetic trafficking (Lippincott-Schwartz et al., 1989). Because we observed that EGFR transferred from ER to Golgi by 4 hr after the temperature shift, we chose to apply the inhibitor at 3 hr. When RBL cells expressing EGFR-GFP are treated with 5μg/ml BFA under these conditions the receptor is not trafficked to the PM (Figure 3.2*A*) but rather is retained intracellularly (Figure 3.2*B*). Evidence that EGFR-GFP is retained in the ER in the presence of

Figure 3.1. *Robust trafficking of EGFR to the PM is observed upon transferring expressing cells to 37℃ following a 14 hr incubation at 24℃.* **(A)** Live cells transiently expressing EGFR-GFP were fixed at each time point. Cells were labeled with an anti-N-terminal EGFR antibody followed by Alexa647-anti-IgG to label PM-associated EGFR, then analyzed by flow cytometry to determine the ratio of PM-localized EGFR fluorescence to total-EGFR fluorescence. Plot is representative of typical results; approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. **(B)** Representative images from time points quantified in **A**.

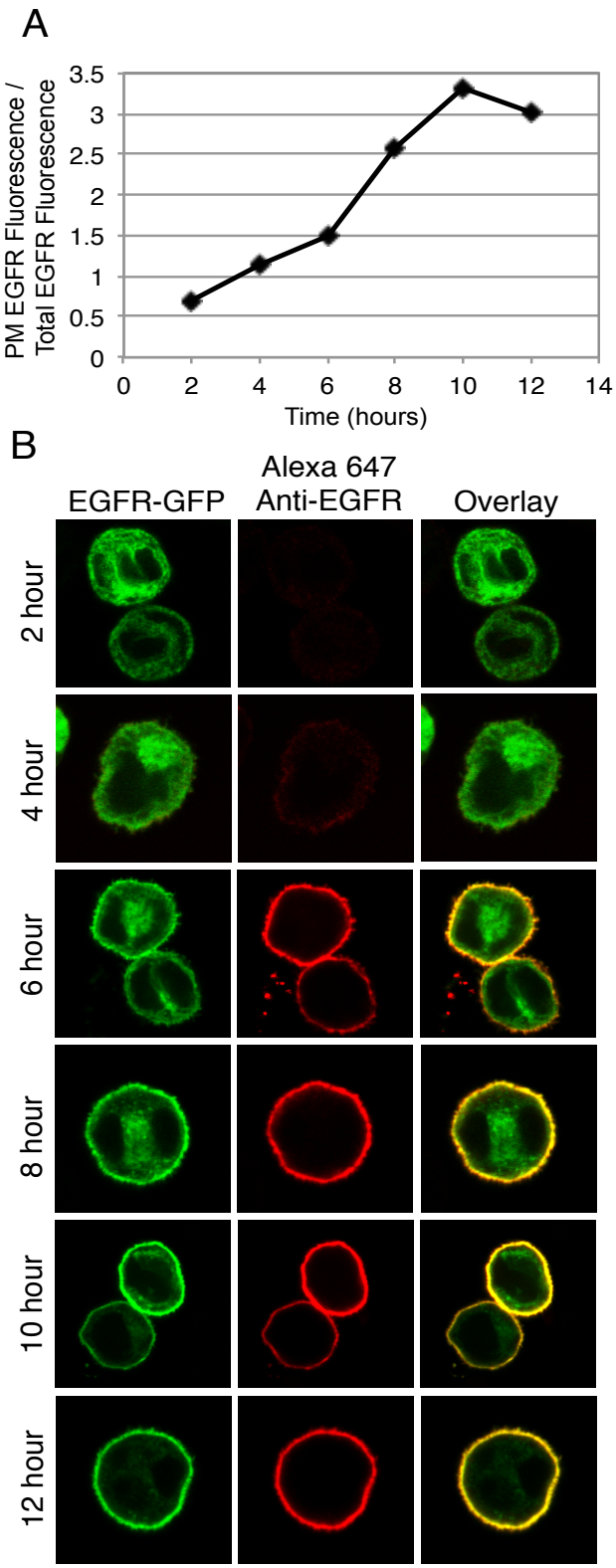
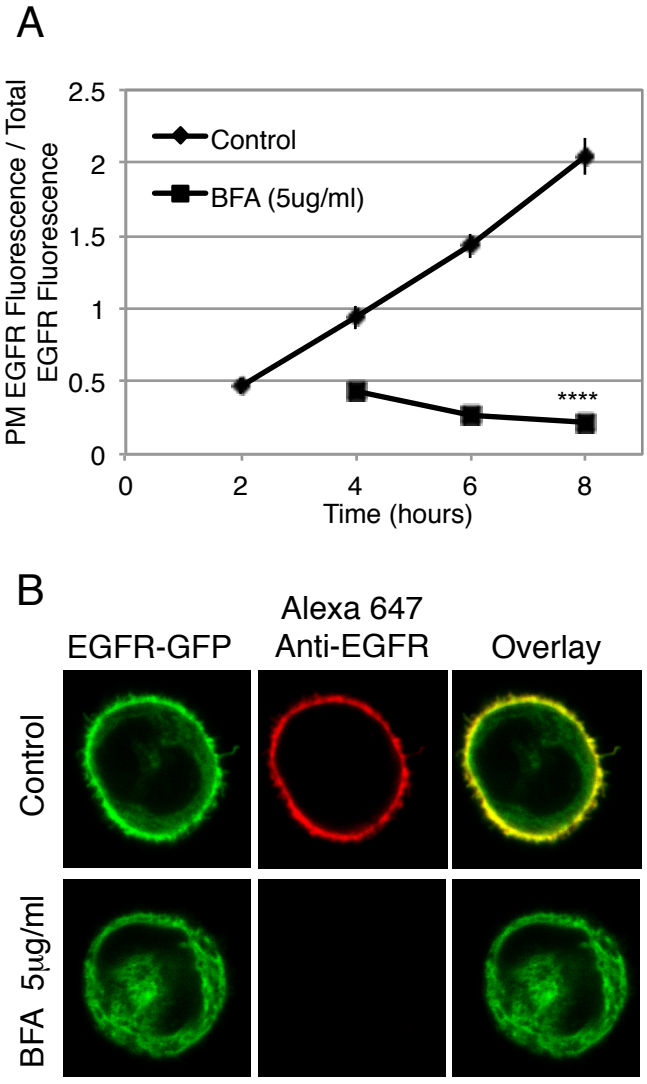

A
PM EGFR Fluorescence / Total EGFR Fluorescence
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
Time (hours)
B
EGFR-GFP
Alexa 647 Anti-EGFR
Overlay
2 hour
4 hour
6 hour
8 hour
10 hour
12 hour

Figure 3.2. *Treatment with Brefeldin A inhibits the biosynthetic trafficking of EGFR to the PM.* Live cells transiently expressing EGFR-GFP were processed as described above (Fig. 3.1), aside from the addition of BFA (5μg/ml) to half of the cells at hour 3. **(A)** Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point ± BFA is plotted. Error bars indicate ± SE of four independent experiments in which approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. ****, $P<0.0001$ **(B)** Confocal images of RBL cells expressing EGFR-GFP either untreated or treated with BFA, cells are from 8 hr time points quantified in **A**.


A
PM EGFR Fluorescence / Total EGFR Fluorescence
2.5
2
1.5
1
0.5
0
Control
BFA (5ug/ml)
****
0
2
4
6
8
Time (hours)
B
EGFR-GFP
Alexa 647 Anti-EGFR
Overlay
Control
BFA 5μg/ml

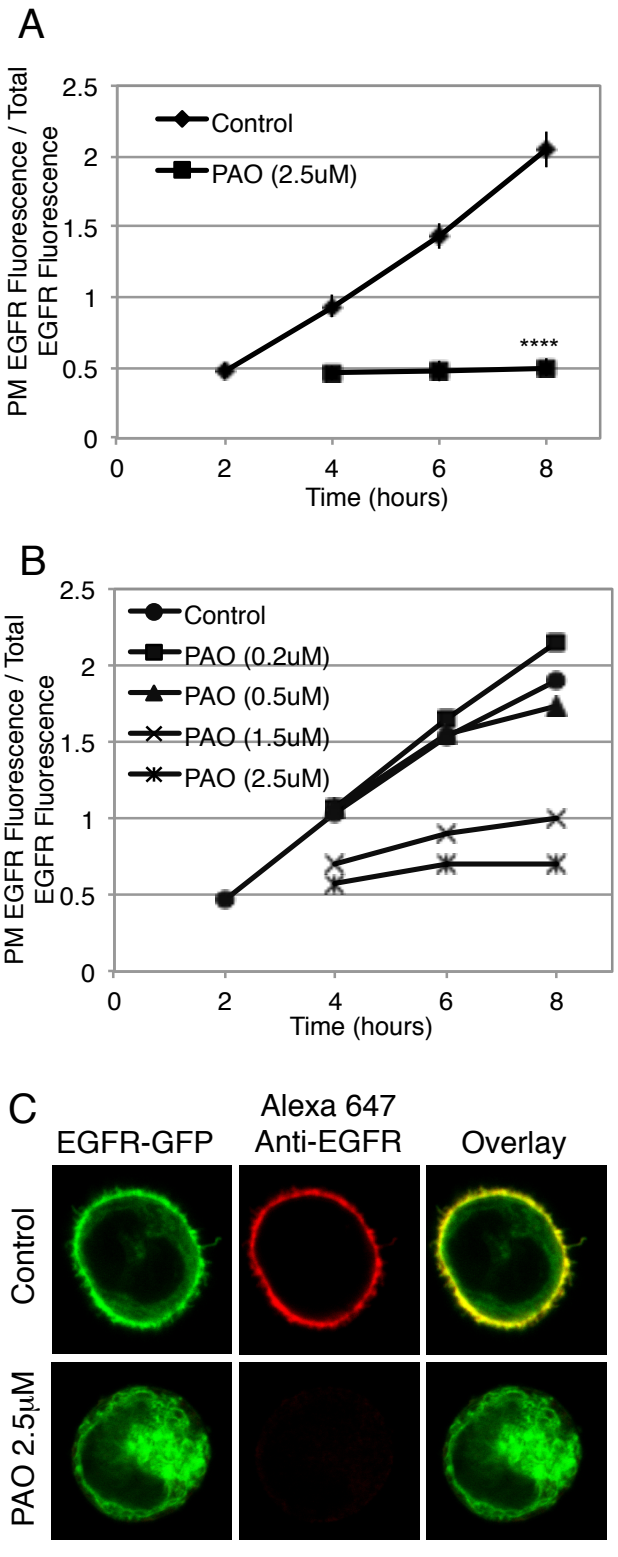
BFA is provided by immunofluorescent colocalization with the resident lumenal ER protein, protein disulfide isomerase (PDI) (Figure 3.6*A*).

3.4.2 Inhibition of PI4KIIIα abolishes the ability of EGFR to traffic from the ER to the Golgi apparatus.

We hypothesized that a pool of PI4P, generated by PI4KIIIα, is necessary for proper biosynthetic trafficking from the ER to the Golgi apparatus. Phenylarsine oxide (PAO) is a well-established inhibitor of PI 4-kinases (Wiedemann et al., 1996), which, when present in low micromolar concentrations, selectively inhibits the PI4KIIIα isoform (Balla and Balla, 2006). When RBL cells expressing EGFR-GFP are treated with 2.5 μM PAO, the receptors are not trafficked to the PM (Figure 3.3*A*) and concentrations as low as 1.5 μM substantially inhibit this biosynthetic trafficking (Figure 3.3*B*). The receptors are retained intracellularly (Figure 3.3*C*), primarily within the ER, coincident with PDI (Figure 3.6*A*).

Although, low micromolar concentrations of PAO specifically inhibit the type-IIIα isoform of PI 4-kinase, PAO can also inhibit tyrosine phosphatases at these concentrations (Garcia-Morales et al., 1990). To determine if the retention of EGFR in the ER upon PAO treatment is due to phosphatase inhibition, we tested the effects of the PI4-kinase inhibitors wortmannin and quercetin [2-(3,4-dihydroxy-phenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one] on biosynthetic trafficking. Neither of these compounds also inhibit tyrosine phosphatases (Graziani et al., 1983; Nakanishi et al., 1994; Santos et al., 2013). Nanomolar concentrations of wortmannin have been shown to inhibit PI3-kinase (IC50=5nM) (Arcaro and Wymann, 1993),

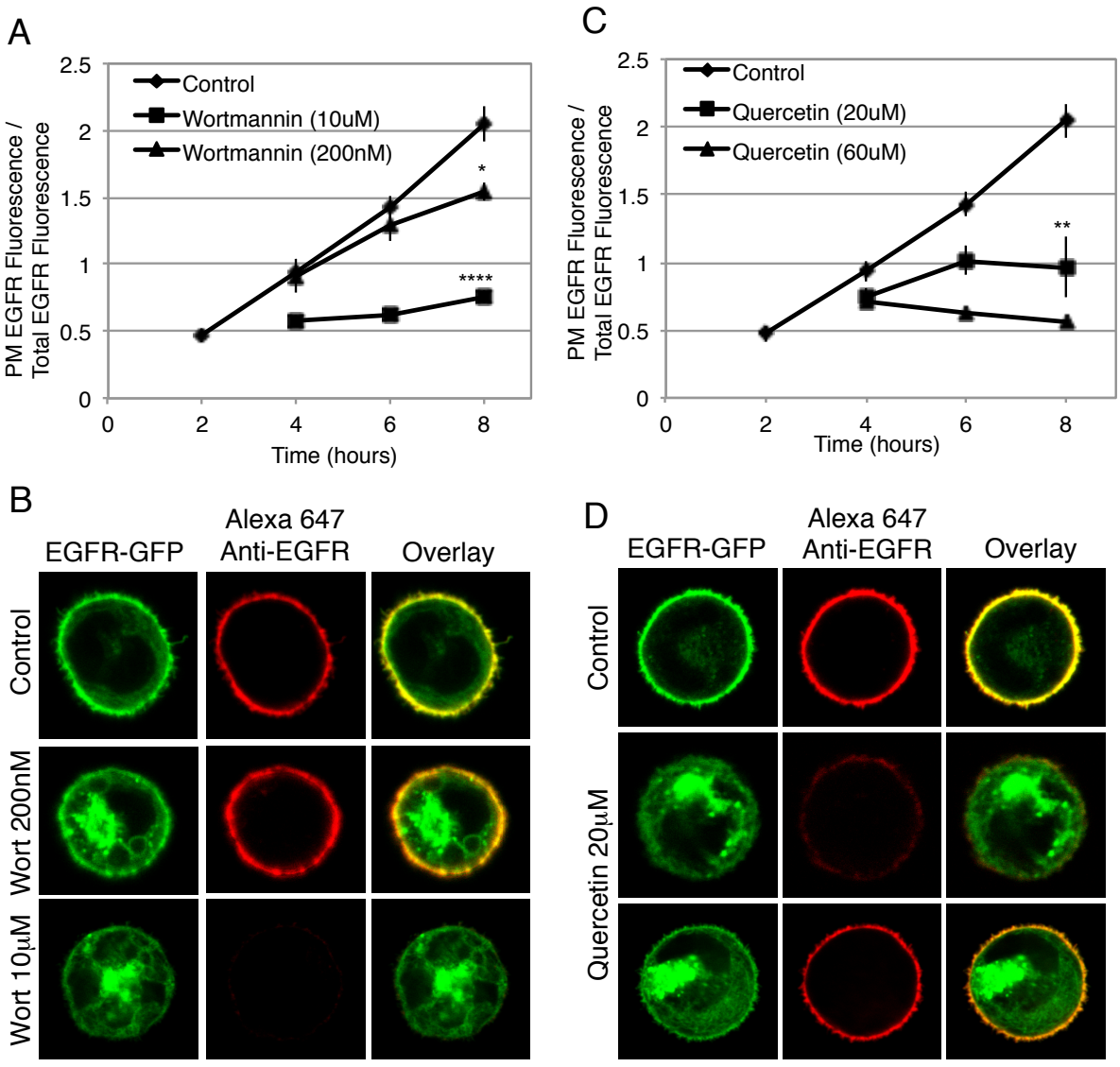
Figure 3.3. *Treatment with PAO inhibits the biosynthetic trafficking of EGFR to the PM in a dose-dependent manner.* Live cells transiently expressing EGFR-GFP were processed as described above (Fig. 3.1), aside from the addition of PAO (concentration defined in legends) to half of the cells at hour 3. **(A)** Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point ± PAO (2.5μM) is plotted. Error bars indicate ± SE of five independent experiments in which approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. ****, $P<0.001$ **(B)** Demonstration of dose-dependent effect of PAO on the biosynthetic trafficking of EGFR. Samples were processed as in **A** following addition of different concentrations of PAO (see legend). Plot is representative of typical results; approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. **(C)** Confocal images of RBL cells expressing EGFR-GFP either untreated or treated with PAO, cells are from 8 hr time points quantified in **A**.


A
PM EGFR Fluorescence / Total EGFR Fluorescence
2.5
2
1.5
1
0.5
0
Control
PAO (2.5uM)
****
0
2
4
6
8
Time (hours)
B
PM EGFR Fluorescence / Total EGFR Fluorescence
Control
PAO (0.2uM)
PAO (0.5uM)
PAO (1.5uM)
PAO (2.5uM)
Time (hours)
C
EGFR-GFP
Alexa 647 Anti-EGFR
Overlay
Control
PAO 2.5μM

whereas significantly higher concentrations are needed to similarly inhibit PI 4-kinases (Downing et al., 1996; Meyers and Cantley, 1997). We found that 10 μM wortmannin abolishes the capacity of EGFR-GFP to traffic to the PM (Figure 3.4*A*), and the receptor is visibly retained intracellularly (Figure 3.4*B*) in both the ER and the Golgi apparatus (Figure 3.6 *A* and *B*). When cells are treated with 200 nM wortmannin, EGFR-GFP biosynthetic trafficking to the PM is significantly reduced (Figure 3.4*A*); but still occurs visibly (Figure 3.4*B*). Quercetin has been shown to generally inhibit kinases, including PI kinases, by competition with ATP for the active site (Middleton et al., 2000). Quercetin treatment significantly reduces biosynthetic trafficking of EGFR in a dose dependent fashion (Figure 3.4 *C*), although some EGFR is detected at the PM by 8 hr when cells are treated with 20 μM quercetin (Figure 3.4*D*). Inhibition of biosynthetic trafficking by PI kinase inhibitors wortmannin and quercetin (Figure 3.4) supports the contention that inhibition of trafficking with PAO (Figure 3.3) is preferentially due to the inhibition PI4KIIIα rather than tyrosine phosphatases.

Organelle-specific PI-kinases and phosphatases dictate distinct subcellular distributions of the individual PI species that control the timing and location of trafficking events (De Matteis and Godi, 2004). Based on immunofluorescence, PI4KIIIα has been suggested to localize to the ER (Wong et al., 1997), but a more recent study indicates a largely cytoplasmic distribution (Nakatsu et al., 2012). Both type-II PI 4-kinases localize to the Golgi complex and endosomes, and PI4KIIIβ localizes more selectively to the Golgi complex (Balla and Balla, 2006). To determine if inhibition of a different isoform of PI4K similarly inhibits biosynthetic trafficking of EGFR from the ER to the Golgi apparatus, we tested the effects of *cis*-Golgi localized PI4KIIIβ inhibition. PIK-93 (phenylthiazole) is a specific inhibitor of PI4KIIIβ (IC50=19nM)

Figure 3.4. *Treatment with the PI4K inhibitors wortmannin and quercetin prevents the biosynthetic trafficking of EGFR to the PM.* Live cells transiently expressing EGFR-GFP were processed as described above (Fig. 3.1), aside from the addition of wortmannin (200nM or 10μM) or quercetin (20μM or 60μM) at hour 3. **(A)** Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point ± wortmannin is plotted. Error bars indicate ± SE of five independent experiments in which approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. *, $P<0.05$; ****, $P<0.0001$ **(B)** Representative confocal images of RBL cells expressing EGFR-GFP either untreated or treated with 200nM or 10μM wortmannin from 8hr time points quantified in **A**. **(C)** Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point ± quercetin is plotted. Error bars indicate ± SE of four independent experiments in which approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. **, $P<0.01$ **(D)** Representative confocal images of RBL cells expressing EGFR-GFP either untreated or treated with 20μM quercetin from 8 hr time points quantified in **C**.


A
PM EGFR Fluorescence / Total EGFR Fluorescence
Control
Wortmannin (10uM)
Wortmannin (200nM)
*
****
Time (hours)
C
PM EGFR Fluorescence / Total EGFR Fluorescence
Control
Quercetin (20uM)
Quercetin (60uM)
**
Time (hours)
B
EGFR-GFP
Alexa 647 Anti-EGFR
Overlay
Control
Wort 200nM
Wort 10μM
D
EGFR-GFP
Alexa 647 Anti-EGFR
Overlay
Control
Quercetin 20μM

(Knight et al., 2006). We found that treatment with PIK-93 does not significantly affect the biosynthetic trafficking of EGFR-GFP, even when used at a concentration (1μM) which should completely inhibit PI4KIIIβ (Figure 3.5 *A* and *B*). To confirm that our PIK-93 stock inhibits PI4KIIIβ we assayed degranulation of RBL-2H3 cells in response to multivalent antigen. We find that PIK-93 reduces this stimulated degranulation by approximately 40% (Figure 3.5*C*), consistent with reduction in degranulation associated with PI4KIIIβ silencing (Kapp-Barnea et al., 2003).

3.4.3 Depletion of PI4P by Sac1 phosphatase abolishes EGFR trafficking from the ER to the Golgi apparatus.

The pharmacological effects described above point to a specific role of PI4P generated by PI4KIIIα in biosynthetic trafficking from ER to Golgi. The only known lipid phosphatases with enzymatic activity towards PI4P are Sac1 homology domain-containing phosphoinositide phosphatases (Guo et al., 1999). The Sac1 protein is a transmembrane protein that localizes to the ER and Golgi membranes and performs organelle-specific roles in lipid signaling (Konrad et al., 2002). Thus, overexpressed Sac1 can serve as a genetic tool to deplete PI4P on the ER and Golgi membranes. We performed our biosynthetic trafficking protocol (Figure 3.1*A*) with cells transfected with both EGFR-GFP and Sac1-GFP, using a three-fold excess of Sac1 or vector DNA to EGFR DNA (Figure 3.7). Sac1-GFP co-expression dramatically reduced the capacity of EGFR-GFP to traffic to the PM (Figure 3.7), further supporting the view that a pool of PI4P is important for ER-to-PM trafficking.

Figure 3.5. *Treatment with PIK-93 does not significantly affect the biosynthetic trafficking of EGFR to the PM.* Live cells transiently expressing EGFR-GFP were processed as described above (Fig. 3.1), aside from the addition of 1μM PIK-93 to half of the cells at hour 3. **(A)** Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point ± PIK-93 is plotted. Error bars indicate ± SE of five independent experiments in which approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point. n.s., not significant **(B)** Representative confocal images of RBL cells expressing EGFR-GFP either untreated or treated with 1μM PIK-93, cells are from 8hr time points quantified in **A**. **(C)** Degranulation is reduced in the presence of PIK-93. Cells were pretreated with denoted concentrations of PIK-93 for 40 min before stimulation with antigen (10 μg/ml) for 25 min. Bars represent average degranulation response from one experiment.

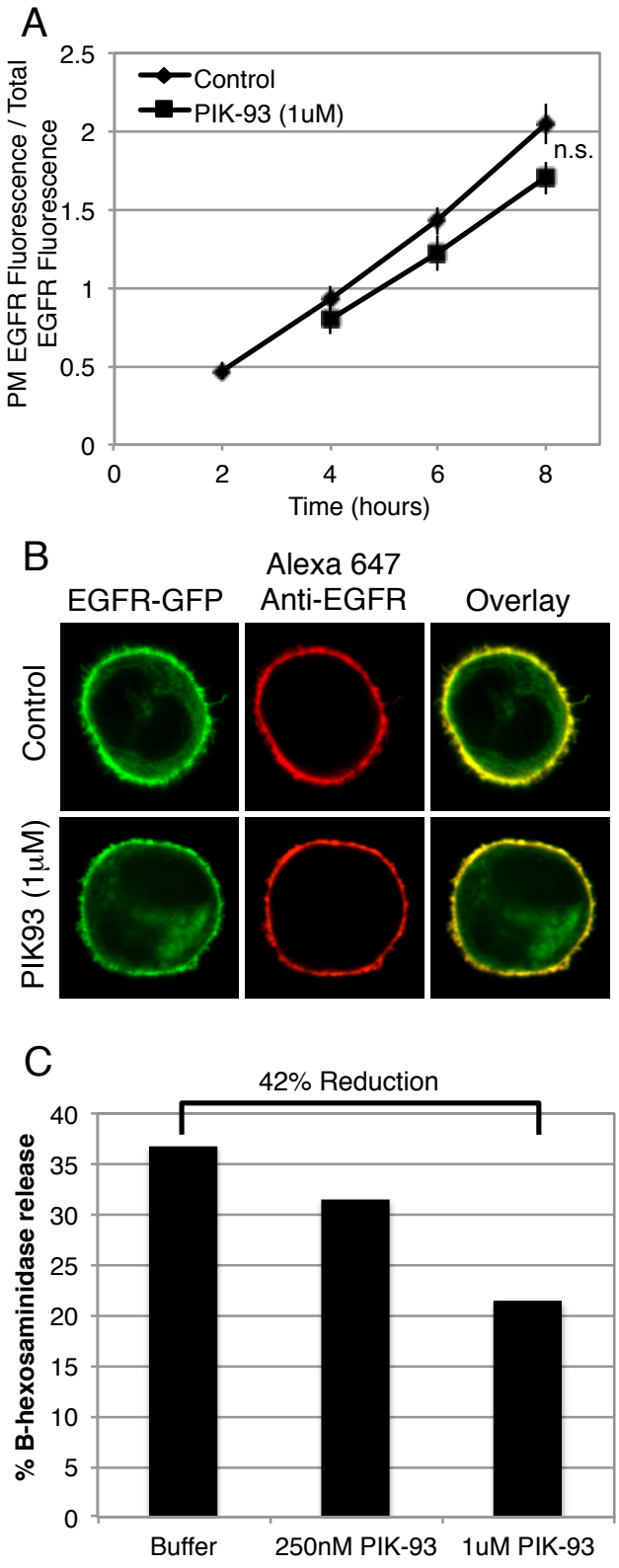
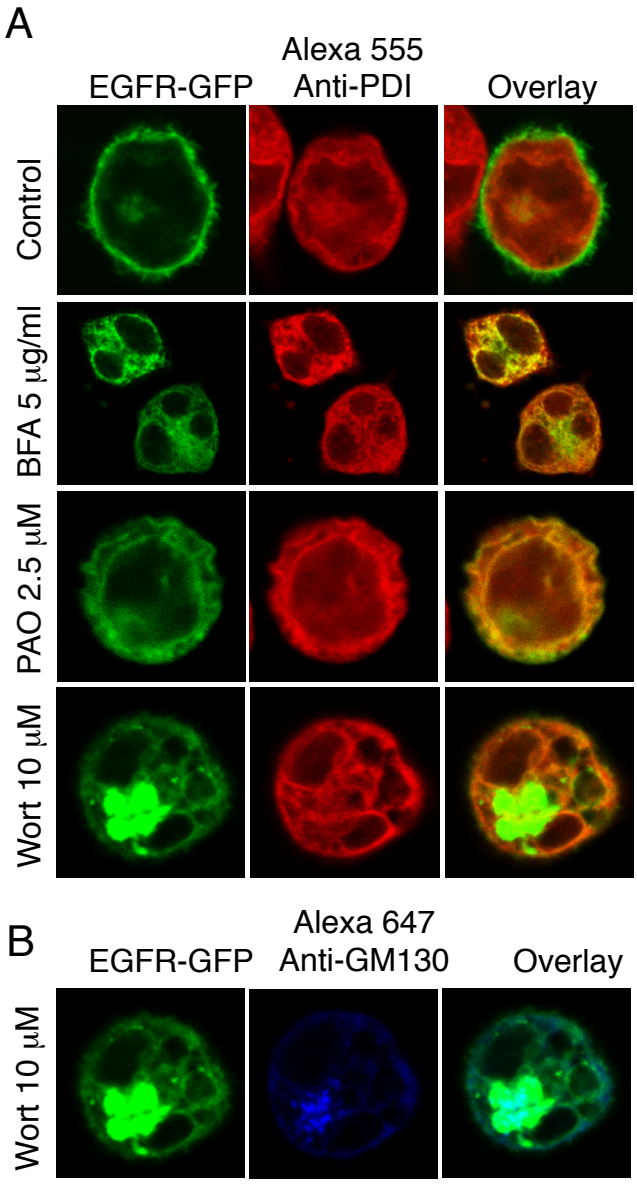

A
PM EGFR Fluorescence / Total EGFR Fluorescence
2.5
2
1.5
1
0.5
0
Control
PIK-93 (1uM)
n.s.
0
2
4
6
8
Time (hours)
B
Alexa 647
EGFR-GFP
Anti-EGFR
Overlay
Control
PIK93 (1μM)
C
42% Reduction
% B-hexosaminidase release
40
35
30
25
20
15
10
5
0
Buffer
250nM PIK-93
1uM PIK-93

Figure 3.6. *Transiently expressed EGFR-GFP is retained within the ER of cells treated with BFA (5μg/ml) and PAO (2.5μM); whereas in cells treated with wortmannin (10μM) retention occurs in the ER and Golgi apparatus.* **(A)** Attached RBL mast cells transiently expressing EGFR-GFP were processed as described above (Fig. 3.1), aside from the addition of inhibitors at hour 3. At hour 8 cells were fixed, permeabilized, and labeled with an anti-PDI antibody followed by Alexa555-anti-mouse IgG to label the ER. **(B)** The same cells shown in **A** were labeled with an anti-GM130 antibody followed by Alexa 647-anti-mouse IgG to label the Golgi apparatus.


A
EGFR-GFP
Alexa 555
Anti-PDI
Overlay
Control
BFA 5 μg/ml
PAO 2.5 μM
Wort 10 μM
B
EGFR-GFP
Alexa 647
Anti-GM130
Overlay
Wort 10 μM

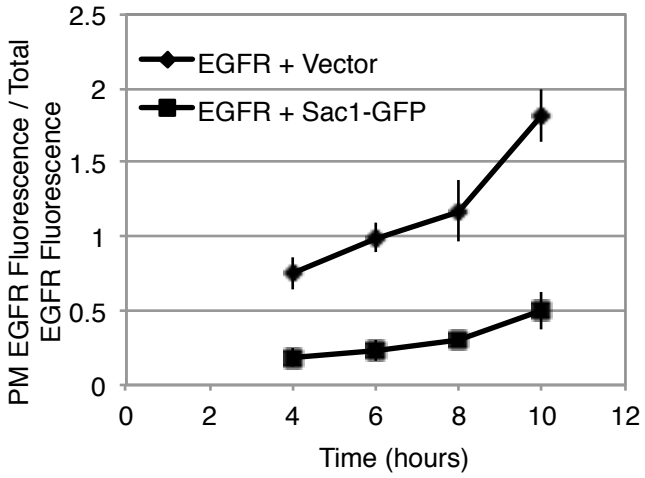


Figure 3.7. *Expression of Sac1-GFP inhibits the biosynthetic trafficking of co-expressed EGFR-GFP to the PM.* Live cells transiently expressing EGFR-GFP and Sac1-GFP were processed as described above (Fig. 3.1). Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point plotted. Error bars indicate ± SD of two independent experiments in which approximately 2,000 double positive cells were analyzed at each time point.

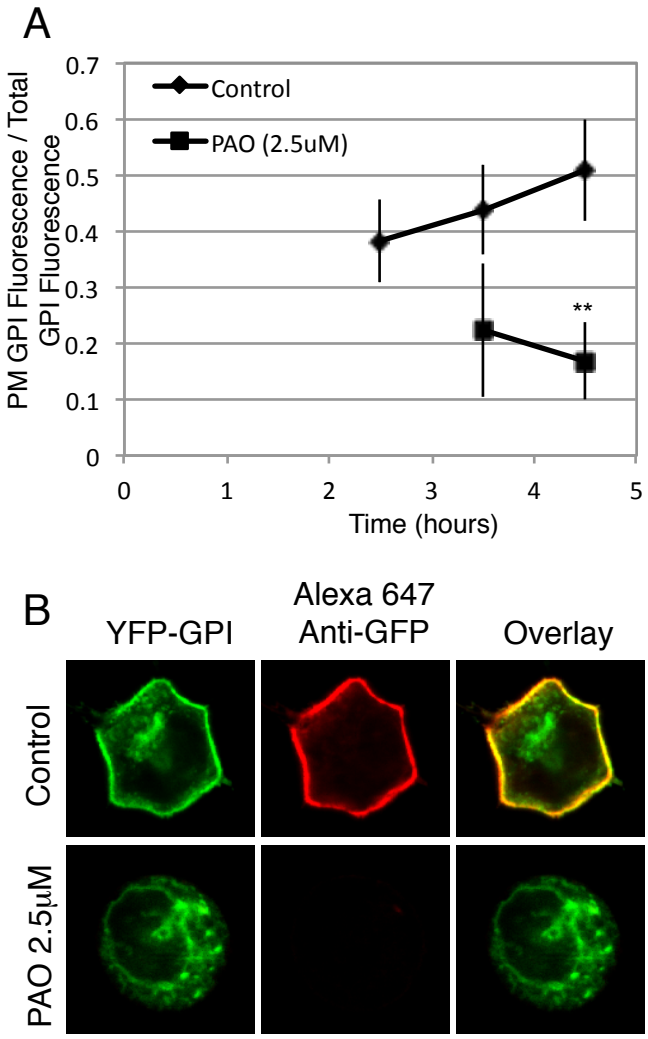
3.4.4 The requirement of PI4P synthesized by PI4KIIIα for ER-to-Golgi trafficking is not limited to EGFR or type-1 transmembrane proteins.

The glycophosphatidylinositol (GPI) anchor is a posttranslational modification that anchors modified proteins to the outer leaflet of the PM (Ferguson et al., 1985). Synthesis of the GPI-linker begins on the cytoplasmic face of the ER; the precursor is then flipped to the lumenal side of the ER for further processing and protein attachment before further trafficking to the PM (Orlean and Menon, 2007). We performed our biosynthetic trafficking protocol with a construct consisting of the GPI anchor of LFA-3, a heavily glycosylated surface protein of broad tissue distribution (Seed, 1987), fused to YFP. In the hours following the temperature shift we observe increased PM presence of GPI-YFP, and this process is inhibited by treating the cells with 2.5 μM PAO (Figure 3.8). This suggests that, PI4P synthesized by PI4KIIIα is involved in ER-to-PM trafficking of two different classes of proteins.

3.5 Discussion

Biosynthetic trafficking of receptors and other membrane proteins from the ER to the PM underlies the capacity of these proteins to participate in crucial cellular functions. Elucidating the mechanisms of the trafficking process is fundamental to understanding and interferring with the cellular responses that PM-localized receptors regulate. Herein, we describe a novel technique to examine the biosynthetic trafficking of transiently transfected proteins. We utilized this technique to reveal the dependence on PI4P, synthesized by PI4KIIIα, for proper biosynthetic trafficking to the PM. We took advantage of a number of well-characterized inhibitors of the different PI 4-kinase isoforms (Balla and Balla, 2006) to demonstrate that the type IIIα isoform synthesizes the relevant pool of PI4P. We complemented our pharmacological studies with a

Figure 3.8. *Treatment with PAO inhibits the biosynthetic trafficking of GPI to the PM.* Live cells transiently expressing GPI-YFP were processed as described for EGFR expressing cells, PAO (2.5μM) was added to half of the cells at hour 3. **(A)** Flow cytometry was used to calculate the ratio of PM localized GPI fluorescence to total GPI fluorescence, and this ratio for each time point ± PAO (2.5μM) is plotted. Error bars indicate ± SD of three independent experiments in which approximately 2,000 GPI-YFP expressing cells were analyzed at each time point. **, $P<0.01$. **(B)** Confocal images of RBL cells expressing GPI-YFP either untreated or treated with PAO, cells are from 4.5 hr time points quantified in **A**.


A
PM GPI Fluorescence / Total GPI Fluorescence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
PAO (2.5uM)
**
0
1
2
3
4
5
Time (hours)
B
YFP-GPI
Alexa 647 Anti-GFP
Overlay
Control
PAO 2.5μM

genetic approach and showed that overexpression of the ER- and Golgi- localized PI4P phosphatase, Sac1, also abolishes protein trafficking to the PM.

The discovery of mutant viral proteins such as vsvg-ts045 (vsvg: vesicular stomatitis virus glycoprotein) allowed for the study of synchronous transport of all of an overexpressed transmembrane protein throughout the biosynthetic pathway in a cell (Bergmann, 1989). In cells transfected with vsvg-ts045, the glycoprotein is synthesized at the non-permissive temperature but is not transported out of the rough endoplasmic reticulum. Upon a shift to the permissive temperature, however, the glycoprotein is rapidly and synchronously transported from the rough endoplasmic reticulum to the Golgi apparatus and then to the PM in a matter of two hours (Bergmann et al., 1981). Here we describe a method that allows for a similar experiment to be performed with any transiently transfected protein. Following transfection, cells are incubated overnight at room temperature. During this incubation, protein synthesis appears to occur without subsequent protein trafficking to the PM. The following day, when cells are shifted to 37°C all of the synthesized protein moves through the biosynthetic pathway. In this study, we monitored the trafficking of EGFR (Figures 3.1 – 3.7) and a model GPI-linked protein (Figure 3.8), both of which were fluorescently tagged and could be labeled with an extracellular epitope binding antibody to visualize the PM associated pool of protein over time. We used flow cytometry, complemented with confocal microscopy, to analyze this process. Biochemical methods, such as endoglycosidase H digestion could also be used with this general approach.

We utilized this method to determine the importance of PI4P for biosynthetic trafficking by pharmacological inhibitors of the different isoforms of PI 4-kinase and by overexpressing a specific phosphatase. We first verified that this system is sensitive to pharmacological intervention by testing the effects of the well-documented inhibitor of early biosynthetic

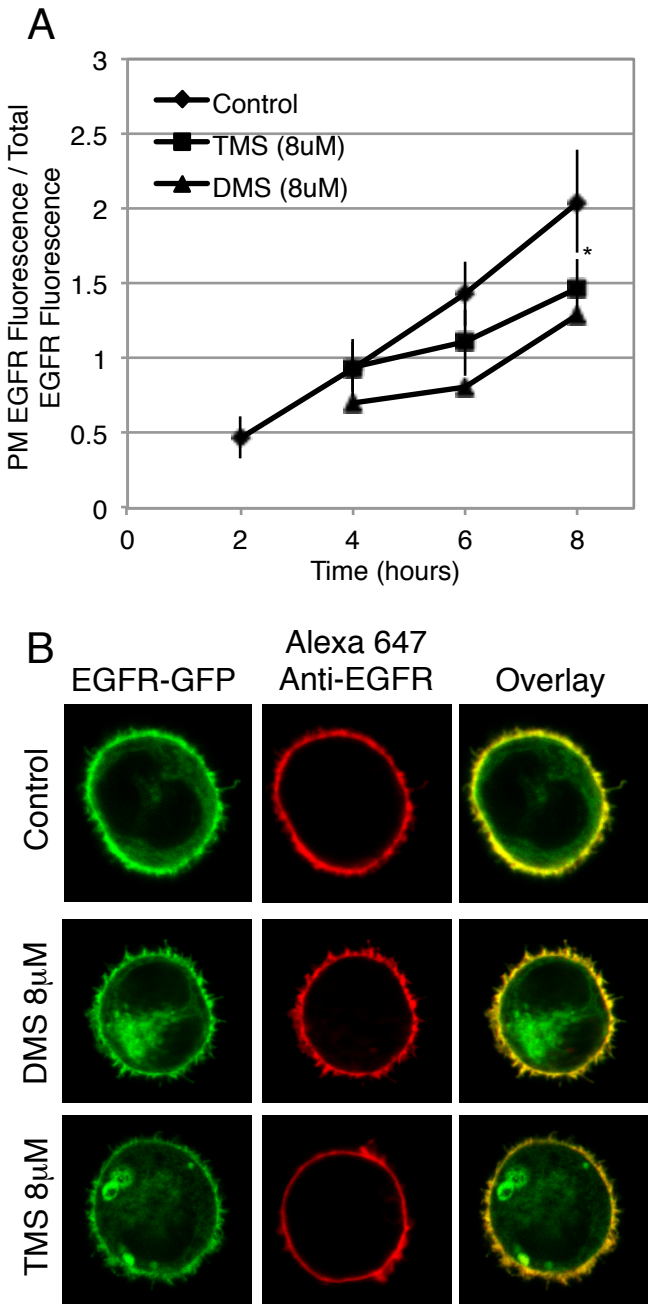
trafficking, BFA (Lippincott-Schwartz et al., 1989) (Figure 3.2). Of the different isoforms of PI 4-kinase, PI4KIIIα is specifically inhibited by low micromolar concentrations of PAO (Balla and Balla, 2006). When cells are treated with PAO in the context of our trafficking method, we observe a dose-dependent inhibition of biosynthetic trafficking of EGFR to the PM (Figure 3.3 *A* and *B*). Maximal inhibition is attained for cells treated with 2.5μM PAO, and EGFR is retained in the ER (Figure 3.6*A*). To test the possibility that inhibition of biosynthetic trafficking by PAO is due to enzymes other than PI4KIIIα, we analyzed the effects of the less specific PI 4-kinase inhibitors, wortmannin and quercetin (Santos et al., 2013). Whereas nanomolar concentrations of wortmannin inhibit PI 3-kinase, wortmannin also inhibits PI 4-kinases at concentrations in the micromolar range (Balla et al., 1997). Quercetin has been shown to inhibit kinases by competing with ATP for binding (Middleton et al., 2000). We observe dose-dependent inhibition of biosynthetic trafficking, when cells expressing EGFR-GFP are treated with either wortmannin or quercetin (Figure 3.4), thus supporting the suggestion that PAO treatment inhibits biosynthetic trafficking by inhibiting PI4KIIIα. Due to the predominant localization of the other three PI4-kinase isoforms to the Golgi, we believe that the perhaps spatially distinct pool of PI4P synthesized by PI4KIIIα may be important for biosynthetic trafficking. This hypothesis is supported by the observation that inhibition of PI4KIIIβ does not significantly affect trafficking of EGFR to the PM (Figure 3.5). Because EGFR must transfer through the Golgi apparatus, we hypothesize that inhibition of one of these three Golgi-localized PI4-kinases is compensated for by the other PI4-kinases localized to this organelle.

We hypothesize that PI4P mediates its effects on the biosynthetic trafficking of proteins via electrostatic interactions with basic stretches of protein sequence. Our laboratory recently showed that long chain sphingosine derivatives such as N,N'-dimethyl sphingosine (DMS) that

flip from the outer leaflet of the PM to its inner leaflet where they are protonated, are effective electrostatic competitors of proteins. The myristoylated alanine-rich C-kinase substrate effector domain (MARCKS ED), which is polybasic and binds to negatively charged phospholipids such as PIP_2, can be competed off the PM with DMS treatment (Smith et al., 2010). In preliminary experiments, we find that DMS is an effective inhibitor of wt EGFR trafficking to the PM, causing EGFR to accumulate in the Golgi apparatus (Figure 3.9). We hypothesize that this accumulation occurs because transport vesicles do not leave the Golgi apparatus to fuse with the PM. Recently, D-sphingosine has been shown to inhibit exocytotic release of insulin and Glut4 expression at the PM of pancreatic beta cells, and a similar electrostatic mechanism of inhibition has been implicated (Pessin et al., 2009). Smith et al. show that N,N'N"-trimethyl sphingosine (TMS) does not flip from the outer leaflet of the PM to the inner leaflet and hence does not electrostatically compete with protein binding (2010). We observe a lesser, yet significant, effect of TMS on appearance of protein at the PM (Figure 3.9), which may be complicated by effects on membrane curvature due to the insertion of the sphingosine and subsequent imbalanced expansion of the outer leaflet of the PM relative to the inner leaflet.

A question currently under investigation in our laboratory is the location of this PI4P pool, which is important for biosynthetic trafficking and synthesized by PI4KIIIα. PI4KIIIα is a cytosolic protein and the subcellular membrane to which it primarily localizes has not been definitively determined (Nakatsu et al., 2013). Based on earlier studies, this enzyme has been reported to localize to the ER (Wong et al., 1997). However, this localization has not been supported by functional studies, which instead point to a role for PI4KIIIα in maintaining a pool of PI4P at the PM (Balla et al., 2008; Nakatsu et al., 2013). In terms of biosynthetic trafficking,

Figure 3.9. *DMS treatment inhibits the biosynthetic trafficking of EGFR to the PM to a greater extent than treatment with TMS.* Live cells transiently expressing EGFR-GFP were processed as described above (Fig. 3.1), aside from the addition of DMS or TMS (both 8μM) at hour 3. **(A)** Flow cytometry was used to calculate the ratio of PM localized EGFR fluorescence to total EGFR fluorescence, and this ratio for each time point ± DMS or TMS is plotted. Error bars indicate ± SD of three independent experiments in which approximately 8,000 EGFR-GFP expressing cells were analyzed at each time point and DMS data represent one experiment. *, $P<0.05$. **(B)** Representative confocal images of RBL cells expressing EGFR-GFP either untreated or treated with 8μM DMS or TMS, cells are from 8hr time points quantified in **A**.


A
PM EGFR Fluorescence / Total EGFR Fluorescence
Control
TMS (8uM)
DMS (8uM)
*
Time (hours)
B
EGFR-GFP
Alexa 647 Anti-EGFR
Overlay
Control
DMS 8μM
TMS 8μM

PI4P localized to the Golgi apparatus has been routinely implicated in the delivery of cargos from the Golgi to the PM (Bruns et al., 2002; Szentpetery et al., 2010). However, a role for PI4P in COPII nucleation at ER-exit sites has also been reported (Blumental-Perry et al., 2006). We observe a primary accumulation of protein in the ER in cells in which PI4KIIIα has been inhibited (Figure 3.6*A*). It is possible that this accumulation is due to depletion of an ER-localized pool of PI4P necessary for exiting the ER, or, alternatively, accumulation could result from depletion of a Golgi-localized pool of PI4P that results in retrograde trafficking to the ER.

We complemented our pharmacological study with a genetic approach to deplete PI4P in the ER and Golgi. We show that overexpression of the Sac1 phosphatase along with EGFR inhibits the biosynthetic trafficking of EGFR to the PM (Figure 3.6). The Sac1 phosphatase cycles between the ER and Golgi apparatus and is a key regulator of PI4P in mammalian cells (Blagoveshchenskaya et al., 2008). Szentpetery et al., described a drug-inducible molecular approach to specifically target the effects of Sac1 phosphatase activity to a particular intracellular membrane (2010). Efforts to employ this technique to deplete PI4P either in the ER or the Golgi apparatus, and thus determine the location of the pool of PI4P important for biosynthetic trafficking, are currently underway.

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Chapter 4

Summary and Future Directions

The juxtamembrane (JX) region of EGFR has recently emerged as a key regulator of receptor function (Red Brewer et al., 2009; Jura et al., 2009; Arkhipov et al., 2013). In November of 2008 we received a shipment from Dr. John Koland that contained an aliquot of EGFR-EGFP. Our laboratory's interest in EGFR had been generated by our finding that mutation of three of the four basic residues within the polybasic JX region of the γ-subunit of a single chain chimeric fusion of FcεRI, completely abolished surface expression (Chapter 1). This result led to the hypothesis that basic residues located in the JX regions of transmembrane proteins play a role in ER-exit, perhaps by interacting with negatively charged lipids. EGFR is a Type I transmembrane protein that contains a polybasic JX region analogous to that of FcεRIγ (Sato et al., 2006). Our studies with EGFR began in order to test the generality of our hypothesis that such basic residues are important for ER to plasma membrane (PM) trafficking. We similarly mutated the basic residues in the JX region of EGFR, and by March of 2009 we already had a result to support our hypothesis. We had generated a mutant EGFR in which six of the nine JX basic residues were mutated to alanines and observed that this mutant was completely intracellularly retained.

While I performed experiments to further characterize the role of the basic JX residues in receptor trafficking, I began to wonder whether my ER-retained receptor was still capable of signaling. The guiding hypothesis for the studies with FcεRI was that reduction of the positive charge in the JX region of the receptor would relieve inhibitory interactions with negatively charged phosphoinositides in the PM and thus allow the receptor to constitutively activate. While

we had originally decided that the ER-retained phenotype precluded a study of the signaling capabilities of the receptor, I became very interested in determining if the ER-retained EGFR was capable of signaling and could transform cells. Following a summer sabbatical in the laboratory of Dr. Cerione, I had NIH 3T3 cells that stably expressed my different EGFR mutants, and on October 19, 2010 I recorded the results of my first anchorage independent growth assay that showed that my ER-retained EGFR JX mutant was capable of forming colonies in soft agar, independent of ligand addition. While I noted that I had only tested one clone in this experiment and would have to test more to confirm the result, the last line on the page of my lab notebook reads, "This could be the start of something beautiful."

In July of 2012, we had written up a draft of a paper that described the beauty that evolved. The receptors were active and transforming cells from their ER locale. They were constitutively phosphorylated and preferentially signaled through the PI3K pathway. In the discussion, we tried to rationalize whether it was the ER-retention or the mutation of the basic residues that caused constitutive activation. As I began experiments aimed at answering that question, I was devastated (at the time) to discover that the JX mutation that I had made did not cause ER-retention. In reality, a random mutation had occurred during the round of mutagenesis that generated my ER-retained mutant, and a single point mutation, L417H, was responsible for the ER-retention.

After I picked myself up and dusted myself off, I went about answering many of the questions that, a few months previously, we thought we couldn't answer. The complete story comprises Chapter 2 of this thesis. While the road to where we are today was rocky, and there was somewhat of an earthquake at the end, it is amazing to think back to November 2008 when it began with an aliquot of EGFR-EGFP. We have acquired a large body of knowledge and

generated a substantial tool set that can be used to study what has become a hot topic in the EGFR field, the role of the JX region in the regulation of EGFR.

In terms of our current contributions to the field, our findings highlight the importance of the polybasic JX sequence in regulating the oncogenic potential of EGFR signaling. We found that charge-silencing mutagenesis within this JX region of EGFR results in the generation of a mutant receptor (EGFR Mut R1-6) that spontaneously transforms NIH 3T3 cells in a ligand-independent manner. We also described a novel point mutation, L417H, which completely retains the receptor in the ER, and showed that the capacity of EGFR Mut R1-6 to mediate transformation is maintained when this mutant is retained in the ER. It is important to note that our study is the first to demonstrate that charge silencing mutagenesis within the polybasic JX region results in spontaneous activity. While others have differently mutated the JX region and shown decreased ligand-dependent activation (Hongbing et al., 2012; He and Hristova, 2012) in agreement with what we demonstrate in Appendix A, they do not report ligand independent activation. This could be due to the fact that their mutations to the JX region are not identical to ours. However, it could also be that they have not done the correct experiments to observe this phenotype. ER-retention and thus, perhaps, protection from PM localized phosphatases dramatically enhances the capacity to detect constitutive phosphorylation of the JX mutants by western blotting. In retrospect, if we had not generated EGFR Mut R1-6 L417H first, the enhanced basal phosphorylation would not have been as easily apparent. Therefore, the soft agar assays, where EGFR Mut R1-6 robustly transforms cells in a ligand independent manner, were critical in discovering this phenotype. To our knowledge, other groups who have recently reported different EGFR JX mutants have not performed such assays.

My work on EGFR has opened up a new vein of research in the Baird-Holowka laboratory

and I hope that future students continue these studies. One potential direction to go is better understanding of the basis for the ER-retention conferred by the L417H mutation. I have begun some preliminary work on this topic. Leucine 417 is located in domain III of the extracellular region of EGFR, which resembles a leucine-rich repeat (Bajaj et al., 1987; Ward and Garrett, 2001). We hypothesized that replacement of leucine 417 with a histidine or alanine residue disrupts the closely packed hydrophobic core of the leucine-rich repeat, and this disruption results in a structural change, which in turn disallows the exit from the ER of EGFRs containing this mutation (Chapter 2). To determine if general disruption of the leucine-rich repeat causes ER-retention, I mutated other hydrophobic amino acids, within this area of the protein that are predicted to interact with leucine 417, including isoleucine 364 and leucine 448. I observed that both EGFR I364A and L448A were largely ER-retained; however, some protein was capable of trafficking to the PM. Thus, we believe that general disruption of this network of leucines can cause ER-retention; nevertheless, residue 417 is the only residue we have found thus far that when mutated completely precludes ER-exit.

These results support the contention that the L417H mutation disrupts the general fold of this region of the protein. I contend that this disruption is local and does not globally unfold the protein. This is supported by the fact that the transformation mediated by EGFR R1-6/L417H is inhibited by gefitinib, as, in order for that to be the case, the kinase domain must be intact. Also, we have shown that the receptors span the ER-membrane and hence have not been trafficked back into the ER lumen for degradation. However, the stabilities of our ER-retained mutants are routinely questioned, and thus I think it is important to determine the half-life of EGFR Mut R1-6/L417H and EGFR L417H as compared to wt EGFR. Prior to leaving, I began these studies by treating cells with cyclohexamide to monitor protein degradation over time and assess whether

the ER-retained mutants are being degraded at a faster rate. Preliminary results suggest that over the course of 10 hours following the simultaneous application of cycloheximide and EGF (100ng/ml), EGFR L417H and EGFR Mut R1-6/L417H are not significantly degraded, whereas both wt EGFR and EGFR R1-6 are virtually eliminated from the cells. While a similar cycloheximide experiment must be performed in the future in the absence of EGF to assess basal degradation, our results thus far support the notion that EGFR L417H and EGFR MutR1-6/L417H are not dramatically destabilized proteins.

Another interesting question that we still do not understand is how EGFR R1-6/L417H becomes activated. Activation is constitutive and clearly is not triggered by ligand-dependent dimerization, as is the case for the PM-localized wt receptor. We are currently investigating the nature of this constitutive phosphorylated state, including the possibility that EGFR Mut R1-6/L417H forms dimers in the ER. Interestingly, two of the mutated residues, arginine 651 and lysine 652, are involved in the formation of an antiparallel coiled-coil that is predicted to be important for the formation of active dimers (Jura et al., 2009). Activation independent of dimer formation has been suggested for EGFRvIII (Chu et al., 1997); thus, understanding the basis for activation of EGFR Mut R1-6/L417H could reveal a potentially novel mechanism for EGFR dimerization that could be more generally relevant for cell transformation by EGFR variants.

To begin in this direction we must first determine whether the EGFR Mut R1-6/L417H dimerizes or not. I have initiated these studies by running unreduced samples on low percentage gels and western blotting for total and phosphorylated receptor. I detect a large molecular weight band that is present and phosphorylated, independent of ligand addition in lysates from cells expressing EGFR R1-6/L417H; this band is also present in lysates from EGF treated, wt EGFR expressing cells (Figure A3 and unpublished results). Thus, we hypothesize that this band

represents an oligomeric, possibly dimeric, EGFR species; however, we have not yet performed experiments in which we can accurately assess its molecular weight. This band decreases significantly in intensity upon reduction of the western blot samples, demonstrating that disulfide bonds are important for maintaining this species. In addition to these studies, Alice Wiesner has recently generated FLAG and HA-tagged wt EGFR and EGFR Mut R1-6/L417H constructs. Using these constructs we expect to demonstrate the presence or absence of dimerization by pulling down one transfected species and blotting for the other in the context of a co-IP.

While the deduction of intracellular signaling cascades is not an easy task, especially when your receptor of interest can trigger many different pathways that can synergize, better understanding the signaling emanating from our JX mutant receptors could provide insight into the plasticity of EGFR signaling. We have shown that EGFR Mut R1-6/L417H exhibits basal tyrosine phosphorylation at residue Y1068 and that this signaling is particularly depends on PI3-kinase and mTOR activity. While we show that EGFR Mut R1-6 mediated transformation is also sensitive to these inhibitors, it is not as sensitive as EGFR Mut R1-6/L417H. I hypothesize that because PI3K and its downstream mediator Akt are cytosolic proteins, which are recruited through protein-protein or protein-lipid interactions, this disposition likely increases their intracellular accessibility for activation by ER-membrane-localized EGFR Mut R1-6/L417H, and thus this pathway is particularly important for transformation mediated by this receptor.

EGFR Mut R1-6 is localized to the PM, and therefore in theory has access to all downstream partners that normally associate with wt EGFR. It is naïve to assume that EGFR Mut R1-6 signaling will be identical to its ER-retained counterpart. I have done some preliminary experiments showing that the JX mutant receptors are not solely phosphorylated at tyrosine 1068; both EGFR Mut R1-6 and EGFR Mut R1-6/L417H also appear to be

phosphorylated at tyrosine residues 845, 992, and 1173. While EGFR Mut R1-6 becomes phosphorylated at all these residues, it is generally less phosphorylated than EGF-stimulated wt EGFR and activates its downstream signaling partners to a lesser extent (Appendix A and unpublished results). The N-terminal halves of the JX region, which include residues that were mutated to alanines to generate EGFR Mut R1-6, have been shown to form an antiparallel helical dimer that stabilizes the formation of the TKD dimer (Jura et al., 2009). We hypothesize that dimerization at least contributes to EGFR Mut R1-6 activation, and therefore propose that the dimers might not be as stable because the receptor lacks certain basic residues. It is also possible that the constitutive activity of EGFR Mut R1-6 (Chapter 2) limits its capacity to mediate a maximal ligand-dependent response. While the level of constitutive activity is low in terms of receptor phosphorylation, this low level of continuous activation mediates robust ligand-independent, anchorage-independent growth (Chapter 2). This capacity must be the result of the constitutive activation of downstream signaling partners, and it is possible that cell-wide constitutive activity changes the biology of the cellular environment in such a way that a maximal response is not possible following a bolus application of ligand. Determining the structural basis of EGFR Mut R1-6 activation and identifying its most immediate downstream signaling partners, independent of ligand, as well as following ligand addition, merits further study.

Historically, the story that became Chapter 3 of this thesis began when we were unaware of the L417H point mutation. At that time, we hypothesized that negatively charged phospholipids, particularly phosphatidylinositol 4-phosphate (PI4P), play an important role in biosynthetic trafficking, at least in part by electrostatic interactions with polybasic sequences often found in the cytoplasmic JX region of transmembrane proteins such as EGFR. To

complement the result that a charge-silenced JX mutant is ER-retained, we wanted to inhibit the production of ER-localized PI4P and similarly retain wt EGFR. Initially, we thought that our hypothesis was correct because treatment with micromolar PAO, which selectively inhibits the potentially ER-localized PI4KIIIα, results in ER-retention of EGFR. However, we realized that there was more to the story when we preformed a biosynthetic trafficking experiment with an EGFR construct in which the polybasic JX region was completely removed, and observed ER-retention when we treated with PAO (unpublished results). This result encouraged us to test the effect of PAO treatment on other classes of proteins, and thus we found that GPI-linked protein trafficking is also inhibited by PAO. In the end, this study supports a dependence on PI4P, synthesized by PI4KIIIα, for proper biosynthetic trafficking of two different classes of proteins to the PM.

My successors could further enhance this story by complementing the pharmacology of Chapter 3 with a genetic approach to deplete PI4P in the ER. We demonstrate that co-expression of the PI-4P phosphatase, Sac1-GFP, and EGFR-GFP in the context of our biosynthetic trafficking protocol inhibits the trafficking of EGFR-GFP (Chapter 3). However, a potential lurking artifact in this experiment is the possibility that merely the expression of another ER-localized protein contributes significantly to the inhibition of trafficking. I have observed this with a number of other constructs and further experiments are necessary to determine if that same artifact is contributing in the Sac1-GFP experiments. Another approach that I have initiated is knocking down PI4KIIIα. There are a number of technical challenges associated with this experiment. These include: the fact that we are using RBL-2H3 cells that notoriously do not transfect well; and the discrepancy between optimal knockdown with siRNA typically occurring 2-3 days post transfection, and the requirement that the protein of interest in a biosynthetic

trafficking experiment be transfected one day prior to the experiment. Potentially, a three-color flow experiment in which siRNA positive cells are marked with one color and two other colors are used to label the total and PM-localized EGFR pools is the answer to this problem; however, more planning and new reagents are required before this experiment can be performed.

I expect that the biosynthetic trafficking assay that we designed in order to perform the experiments described in Chapter 3 will be particularly valuable to others working in this field. While synchronous transport throughout the cellular biosynthetic pathway can be studied using specially designed constructs such as vsvg-ts045 or specialized microscopy techniques; our protocol is unique in that it can theoretically be performed with any fluorescently tagged protein with a detectable extracellular epitope. In the future, we could expand the repertoire of protein classes that can be studied using this technique by attempting to monitor the trafficking of a multi-pass transmembrane protein. We began collecting the necessary reagents for this experiment and thus acquired an Orai1 construct with a HA-tag inserted in an extracellular loop; once this construct is fluorescently tagged, it would be ready to be tested. I used this protocol to demonstrate the dependence of biosynthetic trafficking from the ER to the Golgi on PI4P. In addition, it could be modified in countless ways to study different steps in the biosynthetic pathway as well as the effects of different classes of inhibitors.

In conclusion: Herein, I have described in Chapter 2 the generation of an EGFR mutant in which the net charge of the JX region has been reduced from +8 to +3, due to the mutation of basic residues to alanines. This receptor (Mut R1-6) is constitutively active, and we hypothesize that this is due to the release of inhibitory protein-lipid interactions that hold the receptor in an inactive conformation. I also discovered that a single point mutation, L417H, causes retention of EGFR in the ER, and I showed that when EGFR Mut R1-6/L417H is constitutively expressed it

is capable of transforming cells, in particular by signaling through PI3K and mTOR. In Chapter 3, I demonstrated the importance of PI4P in the biosynthetic trafficking of two classes of proteins. Biosynthetic trafficking of receptors and other transmembrane proteins from the ER to the PM underlies the capacity of these proteins to participate in crucial cellular functions, and this work adds to the body of literature describing the importance of phosphoinositides in regulating this process. In the Appendix, I revisit the EGFR JX mutants and demonstrate that ablating electrostatic interactions of the JX region with the PM changes their capacity for ligand-dependent signaling as compared to wt EGFR.

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Appendix 1

Charge-silencing Mutations in the Polybasic Juxtamembrane Sequence of the EGF Receptor Alters its Capacity for Ligand-Dependent Signaling

A.1 Introduction

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that plays important roles in cell differentiation, proliferation, and epithelial organogenesis (Carpenter, 2000). To maintain normal cellular homeostasis, EGFR signaling must be tightly regulated. Loss or disruption of this regulation can lead to cellular abnormalities such as oncogenic transformation (Blume-Jensen and Hunter, 2001).

EGFR is an integral membrane protein consisting of an extracellular ligand-binding segment, a single transmembrane segment, and an intracellular segment consisting of a juxtamembrane (JX) region, a tyrosine kinase domain (TKD), and a C-terminal tail. Signaling by EGFR is initiated following its ligand-induced dimerization, which leads to TKD-dependent phosphorylation of the C-terminal segments of two interacting receptors (Schlessinger, 2000). The JX region of EGFR has recently emerged as a key regulator of receptor function, and the current model for TKD activation can no longer be discussed without describing its involvement. The TKDs form an asymmetric dimer in which one activates the other, and the C-terminal halves of the JX regions of the receiver TKD form a clasp that promotes this asymmetric activation (Red Brewer et al., 2009; Jura et al., 2009). The N-terminal halves of the JX region that include a polybasic sequence stabilize this interaction via the formation of an antiparallel helical dimer (Jura et al., 2009).

The JX region of EGFR has also been implicated in maintaining the unliganded receptor in an inactive state. Recent evidence suggests that the basic residues in the N-terminal JX region associate electrostatically with negatively charged phospholipids to facilitate insertion of adjacent hydrophobic leucine side chains into the PM bilayer in inactive EGFR. The leucine side chains are then expelled during formation of the helical dimer in the active state (Endres et al., 2013; Arkhipov et al., 2013). In Chapter 2, we demonstrated that reduction of the net charge in the JX region of wild-type (wt) EGFR results in a receptor (EGFR Mut R1-6) that is constitutively active and capable of ligand independent cell transformation.

The phosphorylated tyrosines on the C-terminal tail of EGFR act as docking sites for adaptor molecules that link the receptor to downstream pathways. A multitude of pathways emanate from EGFR including the Ras/MAPK/ERK pathway, the PI3K-Akt pathway, and Ca^{2+} mobilization mediated by PLCγ, which is the focus of this study. Activation of EGFR leads to phosphorylation of the receptor at tyrosine residue 992 and subsequent recruitment of PLCγ (Emlet et al., 1997). Upon phosphorylation and activation, PLCγ hydrolyzes $PI(4,5)P_2$ to form the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1983). IP_3 interacts with the IP_3 receptor in the ER to mediate release of Ca^{2+} from ER stores. Elevated Ca^{2+} binds to calmodulin, which in turn activates Ca^{2+}/calmodulin-dependent protein kinases. Meanwhile, DAG, in concert with Ca^{2+}, activates protein kinase C (PKC). PKC activation leads to phosphorylation and activation of transcription factors (Karin and Hunter, 1995).

Motivated by the hypothesis that a receptor with diminished positively charged residues in the JX region (EGFR Mut R1-6) would have different capacities for signaling in response to ligand, we find that EGFR Mut R1-6 exhibits a reduction in the magnitude of its Ca^{2+} response

compared to wt EGFR. In addition, we show that EGFR Mut R1-6 exhibits reduced phosphorylation of tyr 992 as compared to wt EGFR, corresponding to decreased downstream activation of PLCγ and ERK. While the mechanism for this observation is still under study, we provide preliminary evidence that EGFR Mut R1-6 receptors have reduced capacity to form higher order oligomeric signaling complexes that may be necessary for the optimal activation of downstream signaling partners.

A.2 Materials and Methods

A.2.1 Materials and Plasmids

All cell culture reagents, EGF, and precast gels for blotting were from Invitrogen. FuGene HD was from Roche Applied Sciences. PVDF membranes were from Millipore Corp. The antibodies that recognize the phosphorylated forms of Erk and PLCγ-1 (Y783), as well as phospho-specific EGFR antibody (Y1068), phospho-specific EGFR antibody (Y992), and the anti-EGFR (used for blotting) were from Cell Signaling. The anti-actin antibody was from LabVision/Thermo. HRP-conjugated secondary antibodies used for blotting were from GE Healthcare. The R-GECO-1 was purchased from AddGene.

A.2.2 Cell Culture

Generation of the stable cell lines used for blotting experiment was described in Chapter 2. RBL-2H3 mast cells were grown in MEM containing 20% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals) and 10 μg/ml gentamicin sulfate as described previously (Gosse et al., 2005). Mouse NIH 3T3 cells were grown in DMEM containing 10% (v/v) calf serum (CS).

A.2.3 Intracellular Ca^{2+} Measurements of Suspended cells

In preparation for transfection, cells were plated at 50% confluency into 6-well plates

(Falcon). After approximately 20 h, RBL-2H3 cells were transfected with either mutant or wild-type versions of EGFR, as well as the R-GECO1 Ca^{2+} sensor. These constructs were transfected using Fugene HD (Roche) per manufacturers' instructions, with modification to enhance transfection efficiency in the RBL cells previously described (Gosse et al., 2005). Cells were processed for fluorimetry 24 h after transfection.

Cells were harvested in PBS/EDTA and suspended in buffered saline (BS: 20 mM HEPES, 135 mM NaCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5.6 mM glucose, pH 7.4). Time-based acquisition of cytoplasmic Ca^{2+} responses was monitored using a SLM 8100C fluorimeter, and cells were stimulated with 100 ng/ml EGF. Stimulated Ca^{2+} responses were normalized using total R-GECO1 fluorescence following cell lysis by addition of 0.1% Triton X-100 minus total fluorescence in the presence of excess EDTA as previously described (Vasudevan et al., 2009).

A.2.4 Immunoblot Analysis

After treatment (or not) with EGF (100 ng/ml) for 5min (unless otherwise specified), cells were washed in PBS, incubated in lysis buffer (25mM Tris, pH7.4, 100mM NaCl, 1mM EDTA, 1% (v/v) Triton 100, 1mM DTT, 1mM sodium orthovanadate, 1mM β-glycerol phosphate, 1μg/mL leupeptin, and 1μg/mL aprotinin), and supernatants were retained following microfuge centrifugation. Protein concentrations of the whole-cell lysates (WCL) were determined using the Bio-Rad DC protein assay. WCL (20-35 μg/lane) were resolved by SDS/PAGE, and the proteins were transferred to PVDF membranes. Membranes were blocked by 10% (w/v) BSA in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20, then incubated with the indicated primary antibodies diluted in the same buffer. The primary antibodies were detected with HRP-conjugated secondary antibodies followed by exposure to ECL reagent (Invitrogen).

A.2.5 Data analysis

Analysis and quantification of signal from western blots was conducted using Image J (National Institutes of Health). Statistical analyses were determined using GraphPad Prism (GraphPad Software, La Jolla, CA), using the Student *t* test, with $p \leq 0.05$ considered statistically significant.

A.3 Results

A.3.1 Cells expressing juxtamembrane mutant R1-6 exhibit decreased EGF-stimulated Ca^{2+} mobilization.

To investigate potential roles of basic JX residues in receptor-mediated cell signaling, we focused on the polybasic JX region of EGFR (McLaughlin, 2005; Sengupta 2009). In a previous study, we demonstrated that charge silencing within the JX sequence of EGFR results in constitutive activation of the receptor (Chapter 2). We sequentially mutated basic residues in this sequence to alanines, reducing the net charge of the JX region from +8 to +3 (EGFR Mut R1-6), and we showed that this mutated receptor is basally phosphorylated at tyrosine 1068 and is capable of mediating ligand-independent, anchorage-independent growth. Furthermore, phosphorylation and anchorage independent growth are enhanced in the presence of EGF (Chapter 2).

To investigate the importance of these basic residues in the JX region of EGFR in ligand-dependent signaling, we measured Ca^{2+} levels in suspended RBL-2H3 cells transiently expressing wt EGFR or Mut R1-6 EGFR using the genetically encoded Ca^{2+} probe R-GECO-1 (red intensiometric genetically encoded Ca^{2+}-indicators for optical imaging) (Zhao et al., 2011). RBL-2H3 cells do not express endogenous EGFR, and thus RBL-2H3 cells not ectopically

expressing EGFR do not display an EGF-mediated Ca^{2+} response (data not shown). As shown in Figure A.1 *A-C*, EGF stimulates a biphasic Ca^{2+} response that is significantly larger and faster for the wt EGFR-expressing cells as compared to the Mut R1-6 expressing cells. When multiple experiments are averaged, the Mut R1-6 expressing cells exhibit a peak response that is about 35% of the magnitude of the stimulated wt response (Figure A.1*D*).

A.3.2 Juxtamembrane mutant R1-6 receptors are not efficiently phosphorylated in response to EGF and demonstrate decreased signaling through PLC-γ and ERK.

We investigated whether EGFR Mut R1-6 is phosphorylated in response to EGF and the extent to which it activates the downstream signaling partners necessary for Ca^{2+} mobilization. We observed less EGF-stimulated phosphorylation of EGFR Mut R1-6 by western blotting of whole-cell lysates from NIH3T3 cells stably expressing this mutant receptor, using an anti-phospho-EGFR (Y992) antibody (Figure A.3*A*). This trend is evident in multiple clones expressing similar levels of wt or Mut R1-6 receptor (Figure A.3*A*), and is also observable using an anti-phospho-EGFR (Y1068) antibody (data not shown).

To determine the capacity of Mut R1-6 to signal to downstream partners, we focused our investigation on proteins that interact with EGFR via phospho-Y992, including PLCγ-1. Figure A.2 summarizes results comparing the activity of this protein, as well as ERK, in NIH 3T3 cells stably expressing wt EGFR to those expressing EGFR Mut R1-6. Cells expressing EGFR Mut R1-6 exhibit decreased levels of activated PLCγ-1 (Y783) as compared to wt EGFR expressing cells in response to EGF treatment (Figure A.3 *A* and *B*). Furthermore, decreased levels of activated ERK are also observed in cells expressing EGFR Mut R1-6 as compared to cells expressing wt EGFR (Figure A.2 *C* and *D*). These results provide a consistent picture that the

Figure A.1. *Ca^{2+} responses to EGF for RBL-2H3 cells transiently expressing wt EGFR and EGFR Mut R1-6.* **(A)** Trace shows representative Ca^{2+} response for RBL-2H3 cells transiently expressing wt EGFR stimulated with 100ng/ml EGF from one experiment. **(B)** Trace shows representative Ca^{2+} response for RBL-2H3 cells transiently expressing EGFR Mut R1-6, treated as in **A**. **(C)** Overlay of traces shown in **A** and **B**. **(D)** Quantification of Ca^{2+} response for RBL-2H3 cells transiently expressing wt EGFR and EGFR Mut R1-6. Peak Ca^{2+} responses in the presence of extracellular calcium were averaged from three independent experiments including that shown in **A-C** (error bars show S.D., **, $P<0.01$).

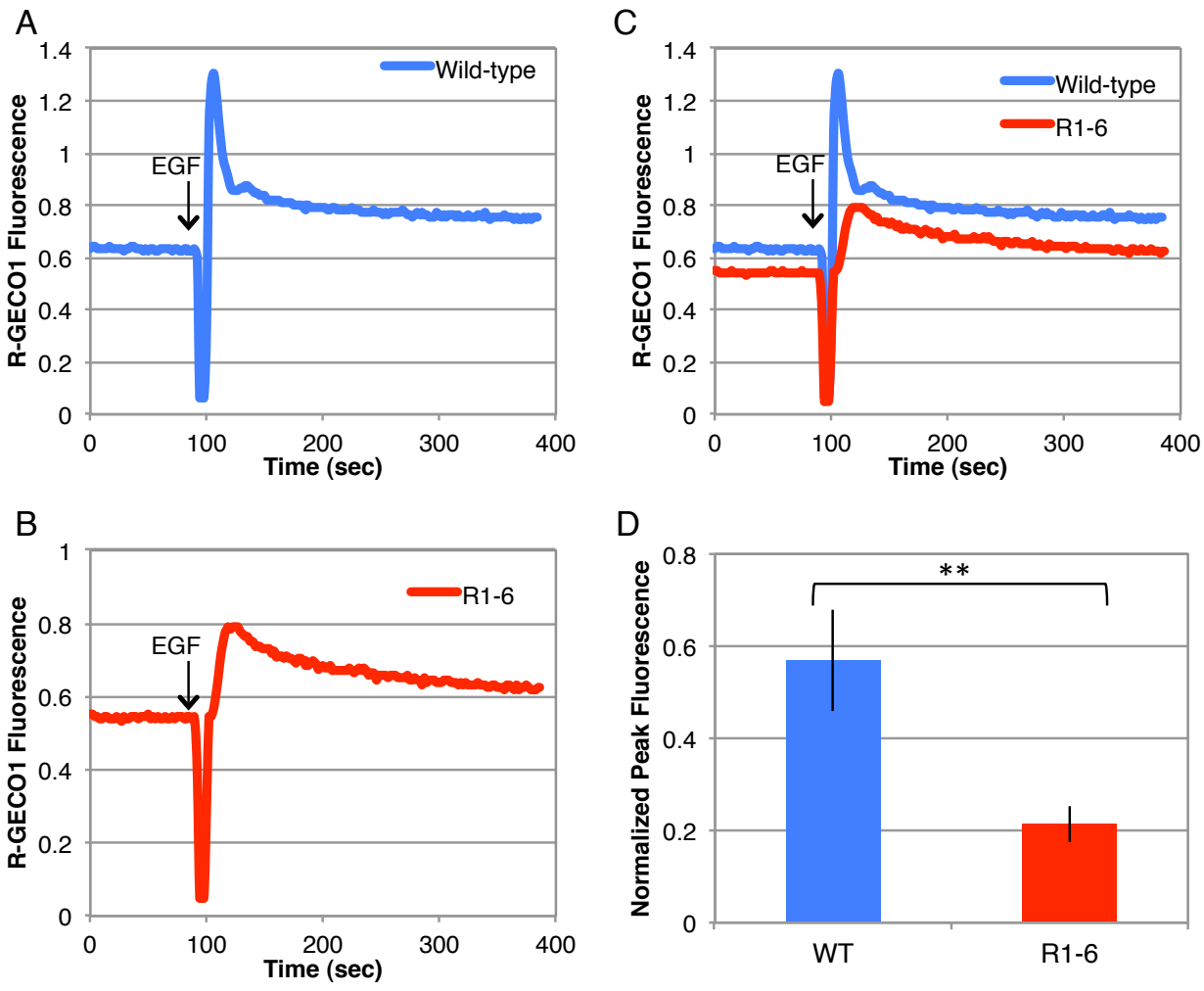
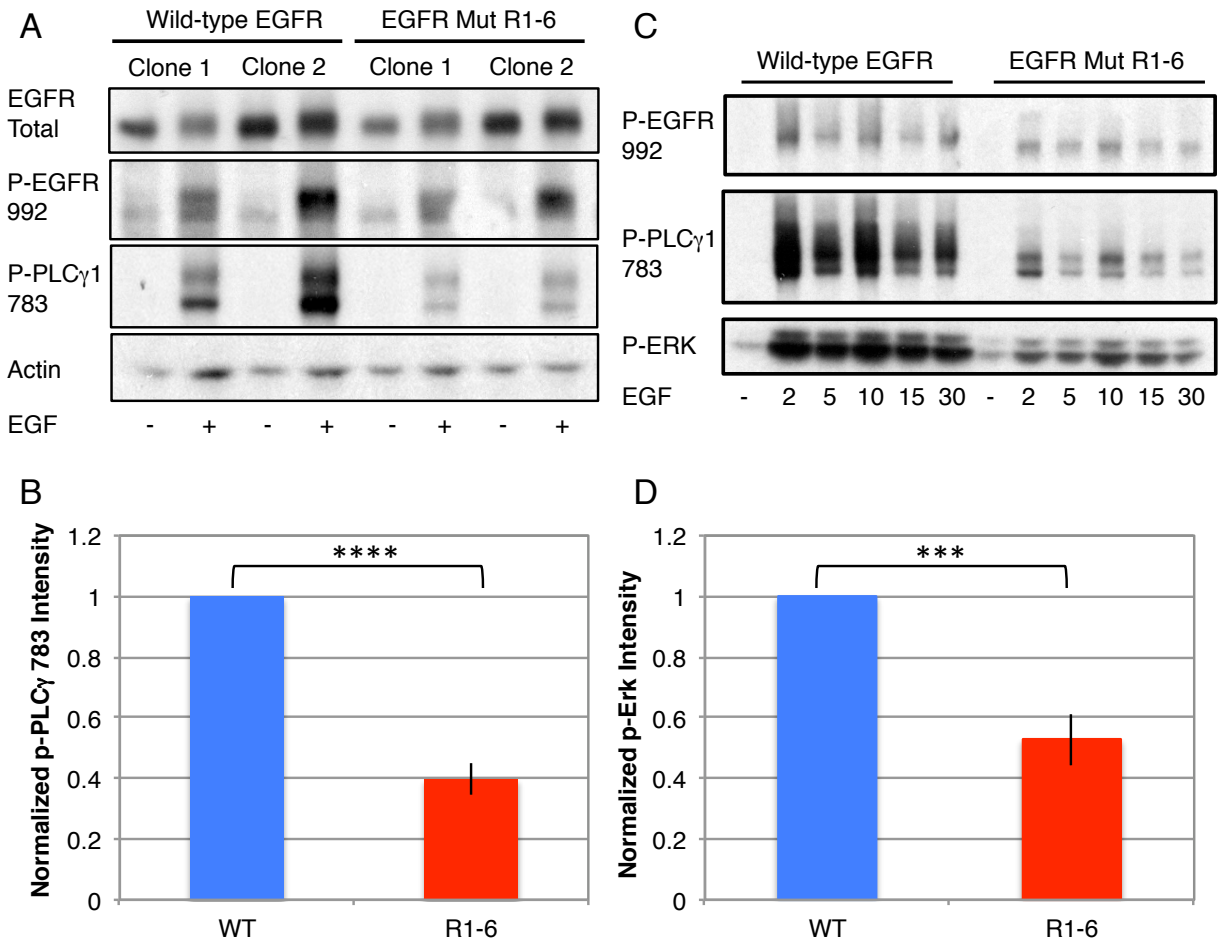

A
Wild-type
EGF
R-GECO1 Fluorescence
Time (sec)
B
R1-6
EGF
R-GECO1 Fluorescence
Time (sec)
C
Wild-type
R1-6
EGF
R-GECO1 Fluorescence
Time (sec)
D
**
Normalized Peak Fluorescence
WT
R1-6

Figure A.2. *EGFR Mut R1-6 receptors exhibit reduced phosphorylation and downstream signaling through PLC-γ and ERK.* **(A)** Whole cell lysates from serum-starved NIH 3T3 cells stably expressing wt or Mut R1-6 EGFR and treated with EGF (100ng/ml) for 5 min as indicated, were immunoblotted with antibodies specific for total EGFR, phospho-EGFR Y992, phospho-PLCγ1 Y783, or actin (loading control). **(B)** Quantification of PLCγ1 phosphorylation in cells expressing wt EGFR or EGFR Mut R1-6 in the presence of EGF for 5 min. Data were averaged from seven independent experiments including that shown in **A** and **C** (error bars show S.E., ****, P<0.0001). **(C)** Whole cell lysates from serum-starved NIH 3T3 cells stably expressing wt or Mut R1-6 EGFR and treated with EGF (100ng/ml) in the context of a time course as indicated (- = no EGF, otherwise time in presence of EGF is shown), were immunoblotted with antibodies specific for phospho-EGFR Y992, phospho-PLCγ1 Y783, or phospho-ERK. **(D)** Quantification of ERK phosphorylation in cells expressing wt EGFR or EGFR Mut R1-6 in the presence of EGF. Data were averaged from five independent experiments including that shown in **C** (error bars show S.E., ***, P<0.001).


A
Wild-type EGFR
EGFR Mut R1-6
Clone 1
Clone 2
Clone 1
Clone 2
EGFR Total
P-EGFR 992
P-PLCγ1 783
Actin
EGF
- + - + - + - +
C
Wild-type EGFR
EGFR Mut R1-6
P-EGFR 992
P-PLCγ1 783
P-ERK
EGF
- 2 5 10 15 30 - 2 5 10 15 30
B
Normalized p-PLCγ 783 Intensity
1.2
1
0.8
0.6
0.4
0.2
0
****
WT
R1-6
D
Normalized p-Erk Intensity
1.2
1
0.8
0.6
0.4
0.2
0
***
WT
R1-6

decreased Ca^{2+} mobilization observed for EGFR Mut R1-6-expressing cells is due to its inability to maximally activate the downstream signaling partners necessary for this response.

A.4 Discussion

The results presented in this Appendix provide evidence that the polybasic JX region of EGFR plays a positive role in ligand-dependent signaling. We have previously reported that reduction of the net charge of the JX region of EGFR from +8 to +3 results in the constitutive activation of the receptor (Chapter 2). Herein, we focus on the response of this mutant receptor (EGFR Mut R1-6) to EGF and its ability to communicate with the downstream signaling partners necessary for Ca^{2+} mobilization. We find that activation of EGFR Mut R1-6 results in a delayed and significantly attenuated Ca^{2+} response (Figure A.1). Consistent with this observation, we show that EGFR Mut R1-6 is sub-optimally phosphorylated in response to EGF and does not activate PLCγ and ERK as efficiently as wt EGFR (Figure A.2).

The ~37-residue JX region of EGFR has emerged as a key regulator of receptor function (Red Brewer et al., 2009; Jura et al., 2009; Arkhipov et al., 2013). In addition to interacting with acidic phospholipids (McLaughlin et al., 2005; Sengupta et al., 2009; Michailidis et al., 2011), several intrinsic sorting signals have been mapped to this JX region (Morrison et al., 1996; He et al., 2002), which also contains a basic calmodulin (CaM) binding sequence (Martin-Nieto and Villaobo, 1998). CaM is a major component of many Ca^{2+}-signaling pathways and mediates the activity of multiple effectors. It has been proposed the CaM directly interacts with the polybasic JX region of EGFR and that ablation of these residues results in decreased activation of PLCγ in response to EGF (Li et al., 2012). We are currently investigating whether EGFR Mut R1-6 has the capacity to interact with CaM.

EGFR Mut R1-6 is not only deficient in its ability to activate downstream signaling partners, these receptors also do not transphosphorylate to the same extent as wt EGFRs. We observed this trend when blotting with antibodies specific for either phosphorylation at tyrosine 992 (Figure A.2) or tyrosine 1068 (data not shown). The N-terminal halves of the JX region, which include residues that were mutated to alanines to generate EGFR Mut R1-6, have been shown to form an antiparallel helical dimer that stabilizes the formation of the TKD dimer (Jura et al., 2009). We have not yet determined whether EGFR Mut R1-6 activation depends on dimerization; however, because its signaling is enhanced in the presence of ligand (Figure 2.5*C*), we hypothesize that dimerization at least contributes to its activation.

We have identified a large molecular weight band that is visible when blotting non-reduced whole cell lysates from cells that stably express wt and Mut R1-6 EGFR (Figure A.3). In wt EGFR expressing cells, the presence of this band depends on the addition of EGF, and it is detectable with both a total EGFR antibody (data not shown) and antibodies specific for EGFR phosphorylated at tyr 992 (Figure A.3) and tyr 1068 (data not shown). We hypothesize that this band represents EGFR dimers or higher order oligomers. Interestingly, while phosphorylation of EGFR Mut R1-6 as represented by the monomer band at approximately 180kDa is reduced as compared to wt (Figure A.2 *A* and *C*; Figure A.3); the signal from this upper molecular weight band is almost entirely abolished in mutant expressing cells (Figure A.3). This band is visible when lysates are probed with an antibody that recognizes total EGFR; however, further quantifiable experiments must be done to determine if the presence of this band is generally reduced in EGFR Mut R1-6 expressing cells. It has been proposed that higher order oligomers and microclusters of EGFR are necessary for maximal activation and robust downstream signaling (Clayton et al., 2007). We are interested in determining if the inability of EGFR Mut

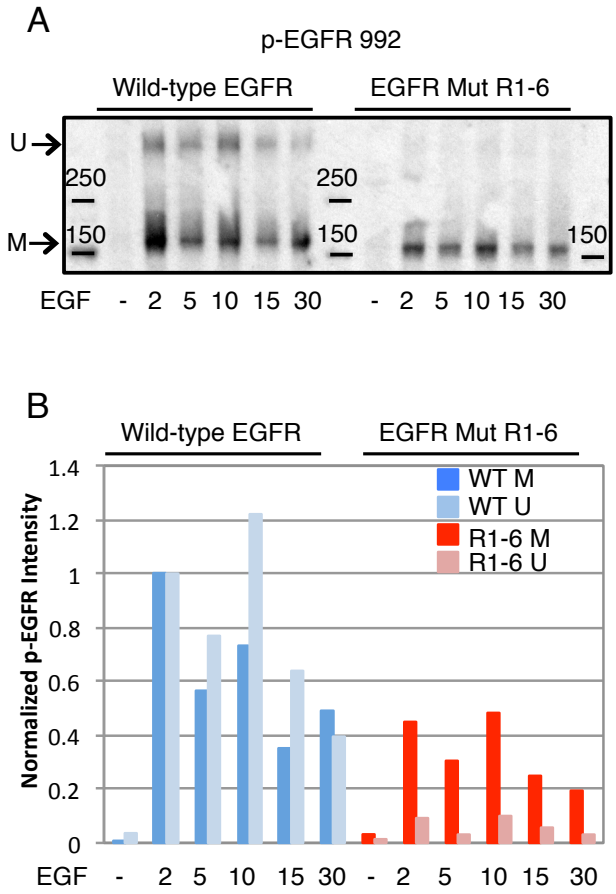


Figure A.3. *EGFR Mut R1-6 receptors exhibit negligible phosphorylation of an upper molecular weight band in response to EGF.* **(A)** Unreduced whole cell lysates from serum-starved NIH 3T3 cells stably expressing wt or Mut R1-6 EGFR and treated with EGF (100ng/ml) as indicated (numbers represent minutes in the presence of EGF) were immunoblotted with an antibody specific for phospho-EGFR Y992. U= upper band and M= monomer band. **(B)** Quantification of phospho-EGFR Y992 intensity from blot in **A**. Data were normalized to the phosphorylation seen 2 min post-EGF addition for the corresponding U and M wild-type EGFR samples. WT= wild type EGFR and R1-6= EGFR Mut R1-6, M= monomer band at ~180kDa, and U= upper band.

R1-6 receptors to form stable dimers or higher order oligomers underlie their reduced capacity for downstream signaling, even in the presence of EGF.

Finally, it is also possible that the constitutive activity of EGFR Mut R1-6 (Chapter 2) underlies it inability to mediate a maximal ligand-dependent response. While the level of constitutive activity is low in terms of receptor phosphorylation, this low level of continuous activation mediates robust ligand-independent, anchorage-independent growth (Chapter 2). This ability must be the result of the constitutive activation of downstream signaling partners. It is possible that cell-wide constitutive activity changes the biology of the cellular environment in such a way that a robust response is not possible in response to a bolus application of ligand. We are currently pursuing this alternative mechanistic explanation for our observations.

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